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EFFECT OF COLD STRESS ON THE PHOSPHORUS METABOLISM OF THE ADRENAL GLAND[1]

BY DORIS NICHOLLS[2] AND R. J. ROSSITER

Abstract

Observations were made on the effects of cold stress (3° ± 1° C.) on the concentration of phosphorus and the incorporation of inorganic phosphate labelled with radioactive phosphorus (P^{32}) into the following P-containing fractions in the rat: the inorganic P of the plasma, the inorganic P, 20-min. hydrolyzable P, and total acid-soluble P of the adrenal gland. With short periods of cold stress (2, 3, 6, 24 hr.) there was an increase in the relative specific activity of each of the adrenal fractions, greatest for an exposure of 2–3 hr. and least for 24 hr., with no significant change in the specific activity of the inorganic P of the plasma. With longer periods in the cold (2, 4, 8, 16 days), in addition to the usual increase in adrenal weight, there was an increase in the relative specific activity of each of the three acid-soluble P fractions of the adrenal. The increase in specific activity was not due to a decrease in the concentration of P in any of the fractions.

Evidence is presented for the view that this change in the phosphorus metabolism of the adrenal can be interpreted in terms of an increase in the rate at which inorganic P^{32} passes across the cell membrane, i.e. from the extracellular to the cellular fluid. It is suggested that the change after a short (2–3 hr.) exposure to the cold is brought about by the stimulation of the adrenal cortex by endogenous ACTH from the pituitary. The change after longer (several days) exposures may be related to the function of the thyroid gland.

Introduction

Since the metabolism of phosphorus is intimately associated with energy-producing reactions, such as may be required during hormone synthesis, an investigation was made of the incorporation of inorganic phosphate labelled with radioactive phosphorus (P^{32}) into the resting and the stimulated adrenal gland of the rat. A reduction in the incorporation of P^{32} into the inorganic P of the adrenal gland of hypophysectomized rats was reported by Gemzell and Samuels (13). These findings were confirmed by Riedel, Logan, DeLuca, and Rossiter (32). Similar observations have been made for the total acid-soluble P (31), lipid P (33), and the nucleotide P of pentosenucleic acid (26). In all instances the incorporation was restored almost to normal by a single injection of ACTH.

[1] *Manuscript received October 18, 1954.*

Contribution from the Department of Biochemistry, University of Western Ontario, London, Ontario. The work was supported by a grant from the Defence Research Board of Canada.

[2] *National Research Council Fellow.*

An increase in the incorporation of P^{32} into the acid-soluble P of the adrenal gland of normal rats exposed to short periods of cold was reported by Reiss and Halkerston (31). From the publication of these authors it is not clear whether the uptake of P^{32} was measured during the time the rats were in the cold room, or after their removal from the cold. In the experiments reported below the uptake of P^{32} was determined both ways, i.e. while the animal was still in the cold room, or after it had been removed to room temperature. The effects of both short (2, 3, 6, and 24 hr.) and longer (2, 4, 8, and 16 days) periods in the cold were investigated.

When the P^{32} uptake took place in the cold room, i.e. while the animal was still exposed to the cold stress, there was an increased incorporation into the acid-soluble P fractions of the adrenal gland. This increase was biphasic. The early response was greater and was maximal after two or three hours in the cold, followed by a return almost to normal. The later phase of increased incorporation did not become maximal until the rats had been exposed to the cold for several days.

When the incorporation of P^{32} was measured at room temperature, after the animal had been removed from the cold room, the relative specific activities of the acid-soluble P fractions of the adrenals of the cold-exposed rats were lower than those of similar fractions from control rats kept at room temperature.

A preliminary report of this work has already appeared (27).

Methods

Animals

Male rats (50–200 gm.) of the Sprague-Dawley strain were injected intraperitoneally with 200 μc. P^{32} as inorganic phosphate* either two or three hours before killing. The animals exposed to the cold were maintained in a cold room at $3° \pm 1°$ C. All animals received unrestricted food (Master Fox Cubes, Toronto Elevators Ltd.) and water. In the experiments reported in Tables I and II the rats were starved for 14 hr. before sacrifice. In view of the report that fed rats failed to show the depletion of adrenal cholesterol normally found after a brief period in the cold (42), a trial experiment was carried out to determine the effect of starvation for 0, 12, 24, or 48 hr. on both control rats maintained at room temperature and rats exposed to the cold for three hours. The relative specific activities of the acid-soluble P fractions of the adrenal glands of the animals exposed to the cold were significantly increased for all groups. Starvation for 0, 12, 24, or 48 hr. produced no significant changes in the phosphate metabolism of the adrenals, or in the response of the adrenal to the three-hour exposure to the cold. The lack of effect of starvation on the changes in P metabolism of the adrenal gland brought about by short exposures to cold thus parallels the ascorbic acid depletion rather than the cholesterol depletion (42). The rats used in the experiments reported in Table III were not starved.

* *Obtained from Commercial Products Division, Atomic Energy of Canada, Limited.*

Extraction of Tissue

A blood sample was obtained from the inferior vena cava under nembutal anesthesia. Heparin was the anticoagulant. The sample was kept in an ice-bath until it was centrifuged in the cold room. The adrenal glands were removed, trimmed rapidly, and immediately frozen in liquid nitrogen. Each pair of adrenals was weighed and homogenized in 2 ml. ice-cold 10% (w/v) trichloroacetic acid (TCA) in a Potter–Elvehjem type homogenizer. The homogenized adrenal tissue was then extracted three times with 10% TCA and the extracts combined. A sample of blood plasma (0.2 ml.) was similarly extracted. The extraction of the acid-soluble P and the subsequent removal of inorganic P were carried out in the cold ($5° \pm 3°$) in order to minimize contamination from the breakdown of labile acid-soluble esters.

Specific Activity Measurements

The specific activity of the inorganic P was determined by the method of Ernster, Zetterström, and Lindberg (11). The specific activity of the 20-min. hydrolyzable P was determined by the same method after hydrolysis for 20 min. at 100° C. with 60% perchloric acid. This fraction, after the values obtained for the inorganic P have been subtracted, consists mainly of the labile P of ATP. The specific activity of the total acid-soluble P was also determined by the method of Ernster *et al.* (11) after preliminary ashing with 60% perchloric acid by the method of King (21).

In the method of Ernster *et al.* (11) both the concentration and the radioactivity of the inorganic P are determined on the same sample. The optical density of the blue color-complex was first read with a Beckman Model B Spectrophotometer at 730 mμ and the radioactivity was then determined on the same colored solution using an M6 liquid counter (20th Century Electronics). The counting rate was corrected for background and decay. The specific activity (counts per min. per μgm. P) of each of the adrenal fractions was expressed as a percentage of that of the plasma inorganic P (relative specific activity). The plasma specific activity for each animal was adjusted to a standard injection of 200 μc./200 gm. body weight, based on a simulated P^{32} standard (Tracerlab Inc., Boston).

Results

Table I shows the specific activity of the plasma inorganic P and the relative specific activity of each of the adrenal fractions after 2, 3, 6, or 24 hr. and after 2, 4, 8, or 16 days in the cold room ($3° \pm 1°$ C.). The specific activity of the plasma inorganic P did not change significantly after 2, 3, 6, or 24 hr. in the cold. There was a significant decrease in the specific activity of the plasma inorganic P after 2 or 4 days, which had returned to normal by 16 days.

In the adrenal gland the relative specific activity of the inorganic P was increased significantly after 2, 3, or 6 hr. in the cold, greatest at 3 hr. After 24 hr. in the cold the relative specific activity of the adrenal inorganic P was not significantly increased ($P > 0.2$). It was significantly increased,

TABLE I

EFFECT OF COLD ON P^{32} INCORPORATION

(Time after P^{32}, 3 hr.; mean ± S.E. mean; figures in brackets give number of animals; P values for test versus control)

Group	Specific activity* (c.p.m./μgm. P)		Relative specific activity × 10^2					
	Plasma inorg. P	*P*	Adrenal inorg. P	*P*	20-min. hydrolyzable P	*P*	Acid-soluble P	*P*
Control	210 ± 4 (84)	—	98.0 ± 2.2 (78)	—	62.1 ± 2.0 (79)	—	57.0 ± 1.7 (83)	—
2 hr. cold†	195 ± 6 (34)	>0.05	107 ± 3.6 (34)	<0.05	63.2 ± 2.9 (34)	>0.7	61.8 ± 2.0 (33)	>0.05
3 hr. cold	202 ± 5 (41)	>0.2	132 ± 3.0 (40)	<0.001	85.9 ± 4.3 (39)	<0.001	86.4 ± 2.4 (40)	<0.001
6 hr. cold	217 ± 9 (20)	>0.4	110 ± 3.4 (20)	<0.01	66.5 ± 4.5 (20)	>0.3	66.0 ± 2.4 (20)	<0.01
24 hr. cold	201 ± 11 (28)	>0.4	104 ± 4.5 (28)	>0.2	76.8 ± 3.1 (27)	<0.001	63.6 ± 2.9 (28)	<0.05
2 days cold	149 ± 3 (12)	<0.001	116 ± 4.0 (12)	<0.001	83.5 ± 3.6 (12)	<0.001	72.2 ± 3.3 (12)	<0.001
4 days cold	153 ± 6 (12)	<0.001	117 ± 5.2 (12)	<0.001	95.7 ± 9.0 (12)	<0.001	76.0 ± 4.3 (12)	<0.001
8 days cold	179 ± 18 (22)	>0.05	116 ± 3.9 (23)	<0.001	86.2 ± 3.6 (22)	<0.001	74.5 ± 2.5 (23)	<0.001
16 days cold	201 ± 12 (13)	>0.4	112 ± 7.0 (13)	>0.05	81.1 ± 6.2 (13)	<0.01	68.5 ± 5.4 (13)	<0.05

** Injected dose, 4.3×10^7 c.p.m. for a 200 gm. rat.*
† P^{32} was injected one hour before the rats were put in the cold.

however, after 2, 4, or 8 days in the cold, but by 16 days the increase was of doubtful statistical significance ($P = 0.1$–0.05). The relative specific activity of the total acid-soluble P was significantly increased after exposures of 3, 6, or 24 hr., but the increase after 2 hr. was of doubtful significance ($P = 0.1$–0.05). The figure for the 20-min. hydrolyzable P was also significantly greater than that for the controls after 3 or 24 hr. in the cold, but not after 2 hr. For each of the experimental periods longer than 24 hr., the relative specific activity of the 20-min. hydrolyzable P and that of the total acid-soluble P were significantly greater than the corresponding figures for the controls.

Dugal and Thérien (8) reported an 11% increase in the adrenal weight of rats kept for 3 days at −1° C., while Woods (46) reported a 33% increase in adrenal weight after 10 days at 0° C. Our results plotted in Fig. 1 are consistent with each of these observations. There was a significant increase in the weight of the adrenal after 4, 8, or 16 days in the cold.

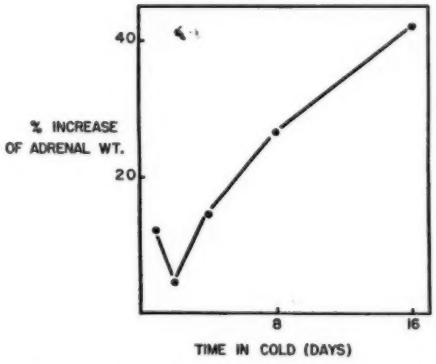

FIG. 1. Effect of exposure to cold (3° ± 1° C.) on the weight of the adrenal gland of the rat.

Table II shows the difference in the results obtained when the P^{32} uptake was studied at room temperature rather than at the temperature of the cold room, for rats exposed to the cold for three hours or for eight days. In the experiments reported in Table I, the P^{32} uptake took place while the rats were in the cold room. The relative specific activity of each of the adrenal fractions in these rats was greater than that of the corresponding fraction in control animals maintained at room temperature. However, when the P^{32} uptake was allowed to take place at room temperature (22° ± 1° C.), 15 min. after removal of the animals from the cold room, there was no increase in the relative specific activities of the adrenal fractions. After three hours in the cold, the specific activity of the plasma inorganic P was significantly lower than that of the controls, while the relative specific activities of the adrenal fractions were not significantly different. Similar results were obtained in other experiments where the animals were exposed to the cold for 2, 6, or 24 hr. and studied after they had been returned to room temperature.

TABLE II

A COMPARISON OF P^{32} UPTAKE IN THE COLD WITH THAT AT ROOM TEMPERATURE

(Time after P^{32}, 3 hr.; mean ± S.E. mean; figures in brackets give number of animals; P values for test versus control)

Group	Specific activity (c.p.m./μgm. P)		Relative specific activity × 10^2					
	Plasma inorg. P	*P*	Adrenal inorg. P	*P*	20-min. hydrolyzable P	*P*	Acid-soluble P	*P*
Control	210 ± 4 (84)	—	98.0 ± 2.2 (78)	—	62.1 ± 2.0 (79)	—	57.0 ± 1.7 (83)	—
3 hr. cold. Uptake in the cold	202 ± 5 (41)	>0.2	132 ± 3.0 (40)	<0.001	85.9 ± 4.3 (39)	<0.001	86.4 ± 2.4 (40)	<0.001
3 hr. cold. Uptake at rm. temp.	158 ± 7 (13)	<0.001	89.4 ± 3.9 (12)	>0.05	58.7 ± 2.8 (12)	>0.3	55.4 ± 4.1 (12)	>0.7
8 days cold. Uptake in the cold	179 ± 18 (22)	>0.05	116 ± 3.9 (23)	<0.001	86.2 × 3.6 (22)	<0.001	74.5 ± 2.5 (23)	<0.001
8 days cold. Uptake at rm. temp.	184 ± 13 (12)	>0.05	75.3 ± 5.4 (12)	<0.001	60.0 ± 5.4 (12)	>0.7	44.4 ± 2.9 (12)	<0.001

Room temperature uptake studies on the rats exposed to the cold for eight days showed that the relative specific activity of the adrenal inorganic P and that of the total acid-soluble P were significantly lower than the corresponding values for the controls. These results indicate that the increased incorporation of P^{32} into the acid-soluble P fractions of the adrenal in animals exposed to the cold is not maintained when the animals are returned to room temperature. This may possibly result from a decreased adrenal activity suitable to the metabolic adjustments associated with a return to room temperature.

In the foregoing experiments, the activities were measured three hours after the injection of the P^{32}, at which time the specific activity of the adrenal inorganic P was almost equal to that of the plasma (i.e. relative specific activity approached 100) in control rats at room temperature. Fig. 2 shows the mean relative specific activity of the adrenal inorganic P in control rats compared with that in animals maintained in the cold for eight days, when measured 2, 3, 6 or 12 hr. after the P^{32} injection. In the controls the specific activity of the adrenal inorganic P approached that of the plasma three hours after the P^{32} injection (relative specific activity approximates to 100). This relationship was maintained for nearly 12 hr. after the injection. In the animals exposed to the cold, the relative specific activity of the adrenal inorganic P reached 100 more quickly, rose above this, and remained greater for more than six hours after the P^{32} injection. Fig. 2 indicates that it would be more advantageous to study the incorporation of P^{32} into the inorganic P of the adrenal at a time interval less than three hours after the P^{32} injection, at which time interval isotope equilibrium would not have been established between the inorganic P of the plasma and that of the adrenal.

In view of the above considerations and the obvious desirability of repeating, under different conditions, experiments such as those reported in Table I,

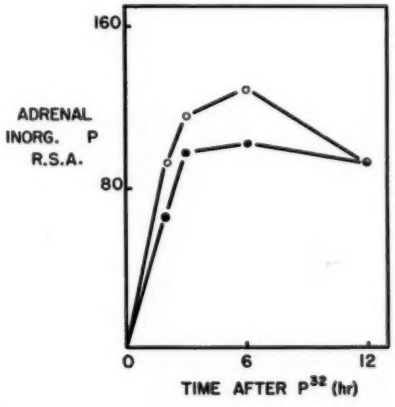

FIG. 2. Relative specific activity (R.S.A.) of the inorganic P of the adrenal gland of rats at different time intervals after injection of P^{32}. ●—●—●, control rats. ○—○—○ rats exposed to cold for eight days.

TABLE III

EFFECT OF COLD ON P^{32} INCORPORATION

(Time after P^{32}, 2 hr.; mean ± S.E. mean; figures in brackets give number of animals; P values for test versus control)

Group	Specific activity* (c.p.m./μgm. P)		Relative specific activity × 10^2					
	Plasma inorg. P	*P*	Adrenal inorg. P	*P*	20-min. hydrolyzable P	*P*	Acid-soluble P	*P*
Control	263 ± 16 (13)	—	65.7 ± 5.0 (14)	—	35.9 ± 3.9 (9)	—	29.3 ± 2.5 (14)	—
2 hr. cold	243 ± 16 (13)	>0.3	116 ± 4.9 (13)	<0.001	68.2 ± 5.2 (11)	<0.001	64.7 ± 3.2 (13)	<0.001
6 hr. cold	268 ± 20 (13)	>0.8	88.0 ± 4.3 (13)	<0.01	48.4 ± 3.2 (10)	<0.05	42.5 ± 1.9 (12)	<0.001
1 day cold	262 ± 21 (13)	>0.9	77.4 ± 4.4 (13)	>0.05	40.2 ± 3.2 (10)	>0.4	31.8 ± 1.8 (12)	>0.4
2 days cold	265 ± 19 (12)	>0.3	78.6 ± 4.6 (13)	>0.05	46.2 ± 5.5 (8)	>0.1	34.4 ± 2.7 (13)	>0.1
4 days cold	264 ± 22 (12)	>0.9	78.8 ± 4.8 (12)	>0.05	42.7 ± 4.2 (10)	>0.2	34.6 ± 2.0 (12)	>0.1
8 days cold	243 ± 16 (10)	>0.4	91.8 ± 7.7 (10)	<0.01	53.0 ± 5.7 (8)	<0.05	39.9 ± 4.7 (10)	<0.05
	Phosphorus concentration (mgm./100 gm. wet weight)							
Control	7.15 ± 0.23 (28)	—	16.8 ± 0.64 (27)	—	22.5 ± 0.87 (27)	—	88.3 ± 1.03 (27)	—
2 hr. cold	7.59 ± 0.28 (14)	>0.2	19.7 ± 1.55 (13)	>0.05	21.3 ± 1.21 (14)	>0.4	88.8 ± 2.14 (14)	>0.8
6 hr. cold	8.84 ± 0.31 (14)	<0.001	17.3 ± 1.19 (14)	>0.7	23.5 ± 1.07 (14)	>0.4	90.6 ± 1.21 (14)	>0.1
1 day cold	8.87 ± 0.25 (18)	<0.001	16.1 ± 0.63 (18)	>0.9	22.9 ± 1.05 (15)	>0.7	94.3 ± 1.66 (17)	<0.01
2 days cold	8.64 ± 0.23 (18)	<0.001	15.5 ± 0.73 (18)	>0.1	22.3 ± 0.99 (14)	>0.8	87.3 ± 2.00 (18)	>0.6
4 days cold	8.07 ± 0.15 (20)	<0.001	14.7 ± 0.62 (20)	<0.02	22.3 ± 0.80 (20)	>0.8	86.7 ± 1.56 (20)	>0.3
8 days cold	7.32 ± 0.26 (12)	>0.6	15.0 ± 0.86 (12)	>0.05	23.0 ± 0.75 (12)	>0.6	90.9 ± 1.49 (12)	>0.1

* *Injected dose, $4{\cdot}3 \times 10^7$ c.p.m. for a 200 gm. rat.*

further experiments were done on the incorporation of P^{32} into the acid-soluble P fractions of the adrenal gland after 2, 6, or 24 hr. and 2, 4, or 8 days in the cold ($3° \pm 1°$ C.). In these experiments the animals were killed two hours, rather than three hours, after the P^{32} injection, the incorporation being allowed to take place while the animals were in the cold. Moreover, the animals were allowed free access to food right up to the time of sacrifice, rather than being subjected to the usual starvation period of 14 hr.

In these experiments (Table III) the specific activity of the inorganic P of the plasma was not changed significantly after any of the periods of exposure to the cold. Table III also shows that the relative specific activity of each of the acid-soluble P fractions of the adrenal was significantly increased after two or six hours in the cold and also after eight days in the cold. Because of the smaller number of animals in these experiments, the increases for animals exposed to the cold for 1, 2, or 4 days were not significant statistically. However, it will be noted that in every instance the mean relative specific activity of the adrenal fraction for these cold-exposed animals was greater than that for the corresponding fraction in the control and, in the case of the inorganic P, the increase approached the 5% level of significance ($P = 0.1$–0.05). More recent experiments have shown significant increases in the relative specific activities of the acid-soluble P fractions of the adrenal gland of cold-exposed rats as little as 30 min. after the exposure to the cold.

A comparison of the figures of Table I with those of Table III shows, as anticipated, that the specific activity of the plasma inorganic P two hours after the P^{32} injection was greater than that found three hours after the

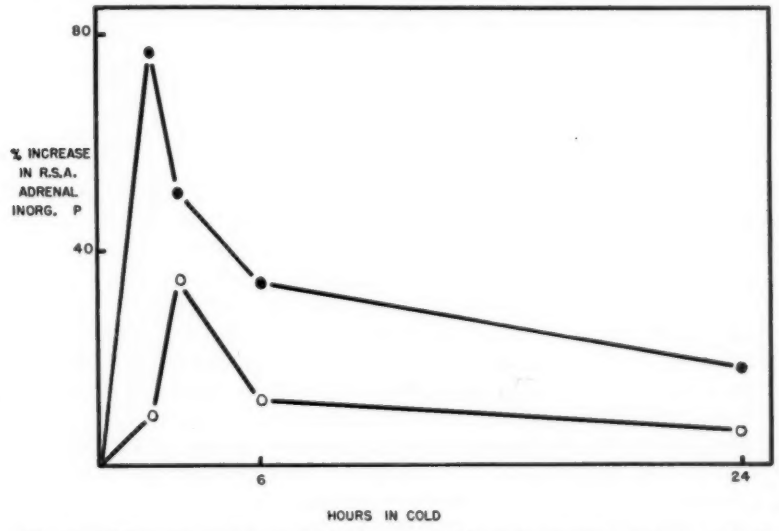

FIG. 3. Effect of exposure to cold (short periods) on the percentage increase in the relative specific activity (R.S.A.) of the inorganic P of the adrenal gland of the rat. ●—●—●, 2 hr. after P^{32}. ○—○—○, 3 hr. after P^{32}.

injection, while the relative specific activities of the acid-soluble P fractions of the adrenal were less when the uptake time was decreased from three to two hours. However, the values for the relative specific activities of the adrenal fractions remained in the same order relative to one another either two or three hours after the P^{32} injection.

Fig. 3 shows the percentage increase in the relative specific activity of the adrenal inorganic P of rats maintained in the cold for 2, 3, 6, or 24 hr., compared with that of control rats kept at room temperature. For all time intervals in the cold, the percentage increase in the relative specific activity

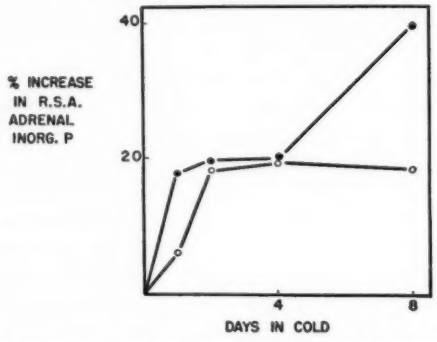

FIG. 4. Effect of exposure to cold (longer periods) on the percentage increase in the relative specific activity (R.S.A.) of the inorganic P of the adrenal gland of the rat. ●—●—●, 2 hr. after P^{32}. ○—○—○, 3 hr. after P^{32}.

of the adrenal inorganic P was greater when the uptake was measured two hours rather than three hours after the P^{32} injection.

Fig. 4 shows similar data for rats maintained in the cold for 1, 2, 4, or 8 days. As in the experiments of a few hours' duration, the percentage increase after cold exposure was greater when the uptake was measured two hours rather than three hours after the P^{32} injection.

Table III also shows the concentration of the plasma inorganic P and of the acid-soluble P fractions of the adrenal gland observed during the experiments discussed above. There was a statistically significant, though small, increase in the concentration of plasma inorganic P after six hours, and 1, 2, or 4 days in the cold, and in the concentration of the adrenal total acid-soluble P after one day in the cold. These changes were not accompanied by significant changes in the specific activities. The concentration of the adrenal inorganic P was slightly decreased after four days in the cold. It is difficult to envisage small changes such as those reported here as being responsible for any great lowering of the pool of unlabelled P, and thereby increasing the ratio of labelled to unlabelled P. It therefore seems unlikely that the increased specific activities described above are due to changes in the concentration of P. Indeed, most of the concentration changes noted here, and in other experiments not reported, are in the direction of an increased rather than a decreased concentration.

Discussion

The Mechanism of the Change in Phosphorus Metabolism

The nature of the alteration in the phosphorus metabolism of the adrenal gland of rats exposed to the cold is of some interest. It is probable that the increase in the relative specific activity of the inorganic P of the adrenal represents a genuine increase in the relative specific activity of the cellular inorganic P. It should be remembered, however, that the relative specific activity of the whole gland a short time after the injection of P^{32} does not necessarily give an estimate of the relative specific activity of the cellular inorganic P, since there may be unequal penetration of the isotope into the extracellular and cellular compartments of the gland. The inorganic P of the whole gland is partly extracellular (plasma and interstitial fluid) and partly cellular.

The difficulties of determining the specific activity of the cellular inorganic P are well known (10, 34). It is widely believed that when inorganic P labelled with P^{32} is administered to an animal, the labelled phosphate rapidly equilibrates with the inorganic P of the extracellular fluid, but that it equilibrates much less rapidly with that of the cellular fluid (15, 19, 20). However, Ennor and Rosenberg (10) have recently questioned the validity of this assumption for rabbit muscle.

In studying the effect of hypophysectomy on the incorporation of P^{32} into the adrenal gland, Riedel and Rossiter (34) concluded that there was a decrease in the relative specific activity of the cellular inorganic P, representing a slowing of the passage of inorganic P across the cell membrane, i.e. from the extracellular to the cellular portion of the gland. By similar reasoning it may be concluded that in the adrenal glands of animals exposed to cold stress there is an increase in the relative specific activity of the cellular inorganic P, representing an increase in the rate at which the inorganic P crosses the cell membrane. The reasons for such a conclusion may be set forth as follows:

1. The relative specific activity of the 20-min. hydrolyzable P was increased after exposure of the animal to the cold (Tables I and III). There is evidence that the 20-min. hydrolyzable P comes into rapid isotope equilibrium with the cellular inorganic P (34).

2. Exposure of the animals to the cold caused a proportionately greater increase in the relative specific activity of the cellular inorganic P calculated by the method of Hevesy (20) than in the total inorganic P (Table IV). Moreover, the increase in the calculated value for the cellular inorganic P produced by exposure of the animal to the cold (two hours or eight days) agreed remarkably well with the increase given by the 20-min. hydrolyzable P (Table V). The figures presented in Table IV were calculated from data given in Table III, based on the assumption that the extracellular fluid compartment comprises either 20% or 36% (28) of the adrenal gland, and that the concentration and specific activity of the inorganic P of the extracellular fluid are the same as those of the plasma.

TABLE IV

COMPARISON OF TOTAL AND CELLULAR INORGANIC P

(Time after P^{32}, 2 hr.)

	Relative specific activity × 10^2		
		Calc. cell. inorg. P assuming extracell. fluid vol. of	
Group	Total inorg. P (from Table III)	20%	36%
Control	66	62	59
2 hr. cold	116	118	119
8 days cold	92	91	90

TABLE V

INCREASE IN THE RELATIVE SPECIFIC ACTIVITY OF CELLULAR INORGANIC P

(Cold/room temperature) × 100

		From calculated rel. sp. activ. assuming extracell. fluid vol. of	
Time in cold	From 20-min. hydrolyzable P	20%	36%
2 hr.	191%	190%	202%
8 days	148%	147%	152%

3. In animals exposed to cold the specific activity of the inorganic P of the whole gland approached that of the plasma (i.e. the relative specific activity approached 100) in less than two hours (Table III; Fig. 2), whereas in the control animals maintained at room temperature the specific activity of the inorganic P of the whole gland did not reach that of the plasma until after three hours (Table I; Fig. 2).

Adrenal Metabolism and Cold Stress

The participation of the adrenal gland in the response of an animal to cold stress was demonstrated by Cannon and associates (3), who showed that there was a secretion of adrenaline from the adrenal medulla following a brief exposure to cold. Cannon (3) postulated that this substance was responsible for the adjustment of metabolism known to take place. That the cortex of the adrenal, as well as the medulla, is involved in resistance to cold is shown by the fact that the administration of cortical extracts (16, 35, 41, 44) or desoxycorticosterone (23) increases the survival time of adrenalectomized rats exposed to cold.

The findings reported here confirm the original report of Reiss and Halkerston (31). In addition, a study now has been made on the effects of cold stress of either short or long duration on the incorporation of P^{32} into three acid-soluble P fractions of the adrenal gland and into the inorganic P of the plasma. When the P^{32} uptake is measured in the cold, there is an increased incorporation into the adrenal that occurs in two phases: (1) the early phase, maximal after two or three hours in the cold, and (2) the later phase that is evident after several days in the cold. The suggestion that there may be both an immediate and a later endocrine response to a cold environment is not new (see references quoted below).

The Early Phase

An increase in the incorporation of P^{32} into the acid-soluble P fractions of the adrenal gland is a constant finding in animals exposed to cold for short periods of time. Similar short periods of cold stress also cause both an increase in the function of the medulla of the adrenal, as shown by the liberation of adrenaline into the circulation (3), and an increase in the function of the cortex of the adrenal, as shown by the well-known depletion of adrenal ascorbic acid and cholesterol (25, 36).

Since exogenous ACTH also causes an increase in the incorporation of P^{32} into the acid-soluble P fractions of the adrenal (13, 31, 32), it seems reasonable to suppose that the changes in phosphorus metabolism that follow short periods of exposure to the cold are a direct result of the stimulation of the cortex of the adrenal gland by endogenous ACTH from the pituitary.

Previous studies have shown the importance of the adrenal gland for the survival of an animal during the early period of cold exposure. For example, removal of the adrenals is followed by the rapid death of non-acclimatized rats exposed to cold (6, 23, 38, 40).

In addition to the adrenal, the thyroid gland may play an important role during the early response of an animal to cold. If the thyroid gland is hyperactive, the increased production of thyroid hormone may cause an increased uptake of P^{32} into the acid-soluble P of the adrenal, just as the hyperthyroid condition is responsible for an increased incorporation of P^{32} into the acid-soluble P fractions of muscle (14) and liver (45). Leblond and Gross (23) showed that a minimum of thyroid hormone was necessary during the early phase of cold exposure. This finding was confirmed by Sellers *et al.* (38), who reached a similar conclusion from a study of the effect on survival of changing the time interval between thyroidectomy and cold exposure. Other experiments indicate an increase in either the requirement of thyroid hormone or in the activity of the thyroid gland, even during the early stages of exposure to cold (2, 22, 43).

The Later Phase

In animals adapted to cold, removal of the adrenal glands has not so drastic an effect (6, 23, 38, 40). Selyé (40) has interpreted this as an indication that the hormones of the adrenal cortex are not essential at this stage. On the

other hand, the reports of a number of workers would seem to imply that, although adrenal hyperfunction may not be essential, a certain level of adrenal activity is still necessary (6, 17, 18, 23, 38). This is borne out by the prolonged changes in adrenal weight and cellular hyperplasia (1, 8, 9, 39, 40, 46), by the changes in staining properties (7, 12), and by the increase in certain enzyme activities in the gland (5, 29).

As seen above, the increased incorporation of P^{32} into the acid-soluble P fractions of the adrenal during short periods of cold stress is thought to be due to the stimulation of the adrenal cortex by the liberation of endogenous ACTH. However, it is less certain whether the increased incorporation after longer periods of exposure to cold is brought about by the pituitary–adrenal mechanism. A number of workers have stressed the importance of the thyroid gland to animals exposed to cold for longer periods of time (4, 22, 23, 24, 37, 38). Good evidence in this regard is the finding of an increased thyrotrophic activity of the pituitary by Sellers and You (37) and the report of Leblond and Eartly (22) that thyroxine-deficient animals require more thyroid hormone when maintained at low temperatures. There is also evidence that animals treated with thiouracil require more thyroid hormone when exposed to the cold (4, 30).

Experiments designed to assess the importance of a normally functioning pituitary and thyroid in both the early and later phases of the adrenal response are in progress.

Acknowledgment

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ASSAY OF THYROTROPHIN BY THE ACCUMULATION OF RADIOACTIVE IODINE IN THE THYROID OF THE INTACT RAT[1]

By N. R. Stephenson, W. P. McKinley, and P. J. Kavanagh

Abstract

The ability of the thyroid gland of intact weanling rats to collect injected radioactive iodine was diminished by inclusion of a small amount of iodinated casein in the diet. The administration of exogenous thyrotrophin increased the thyroidal uptake of I^{131} in rats treated in this manner. Both the degree of inhibition of the thyroid and the ability of the animals to differentiate between dosage levels of thyrotrophin were dependent upon the amount of iodinated casein in the diet. Graded doses of thyrotrophin provided a linear log dose – response line over the range 0.01 to 0.08 U.S.P. units. The response approached a maximum value at dose levels above 0.16 U.S.P. units. The thyroid gland of the female weanling rat retained, on the average, significantly more radioactive iodine than that of the male weanling rat when stimulated by thyrotrophin. Although the hormonal response was not correlated with the body weight of the rats within dosage groups, more precise assays were obtained by equalizing the total weight of the rats in each dosage group before the assay. Relatively large doses of I^{131} appeared to have an adverse effect on the precision of the assay but did not influence the response to thyrotrophin.

It is well known that the uptake of radioactive iodine (I^{131}) by the thyroid is influenced by the thyrotrophic hormone of the anterior pituitary gland (7, 8, 15). Ghosh, Woodbury, and Sayers (4) studied the quantitative effects of thyrotrophin on the accumulation of radioactive iodine in the thyroid of hypophysectomized rats, and demonstrated that it was possible to develop a useful assay procedure employing such a criterion of the response to the hormone. Brimblecombe and co-workers (2) investigated several methods for the determination of thyrotrophin and concluded that the thyroidal uptake of radioactive iodine in the hypophysectomized rat provided the most precise assay of those examined.

The administration of either iodide, desiccated thyroid, thyroxine, or iodinated casein has been shown to inhibit the ability of the thyroid gland to accumulate radioactive iodine (5, 6, 9, 11, 13, 16). Overbeek *et al.* (9) were able to lower the thyroidal uptake of I^{131} to levels usually found in hypophysectomized rats by feeding a diet containing 50 mgm. of iodinated casein per kgm. for approximately 12 days. The subcutaneous injection of graded doses of thyrotrophin to these pretreated rats increased the capacity of the thyroid to collect I^{131} in such a way as to produce a linear dosage–response relationship over the dose range of 0.01 to 0.16 U.S.P. units of thyrotrophin. The index of precision of the assay described by Overbeek and co-workers (9) was of the same order of magnitude as that reported by Ghosh, Woodbury, and Sayers (4) using hypophysectomized rats.

The work described in this communication has confirmed the observations made by Overbeek *et al.* (9). The thyroidal uptake of I^{131} by intact weanling rats pretreated with iodinated casein was found to serve as a satisfactory criterion of the response to exogenous thyrotrophin.

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Experimental

Male and female rats of the Wistar strain, 21 days of age and weighing 30 to 40 gm., were fed a diet* containing amounts of iodinated casein† ranging from 30 mgm. to 200 mgm. per kgm. for 12 days during a pretreatment period. Each rat consumed approximately 5 gm. of the diet per day during this time.

Before the administration of the thyrotrophin, the animals were divided at random into dosage groups containing 6 to 10 rats each. The total weight of the animals in each group was equalized by appropriate exchange after the original random distribution. To each dosage group was allotted the same number of rats of each sex.

The thyrotrophin preparation was dissolved in 0.9% saline and administered in two divided subcutaneous injections, the first at 9 a.m. and the second at 5 p.m. At least two dose levels of the hormone were given, employing a log dose interval of 0.301. At 9 a.m. on the following day, each rat received an intraperitoneal injection of 0.5 ml. of isotonic saline containing approximately 2 microcuries of carrier-free radioactive iodine (I^{131}). The animals were then placed in cages on wire netting for 24 hr. during which time they were given neither food nor water. At the end of this period, the rats received an overdose of chloroform and the portion of the trachea with the entire thyroid gland attached to it was removed and dropped into a 2 *N* sodium hydroxide solution containing 1% potassium iodide and 0.1% sodium bisulphite (10). The volume of this solution varied from 2 to 4 ml. depending directly upon the dose of thyrotrophin given to the rat. The thyroid tissue dissolved when allowed to stand in the solution overnight at room temperature. A 1 ml. aliquot of the digestion mixture was placed in a stainless steel planchet and the number of counts per min. was recorded by means of mica end-window Geiger-Mueller counter (3). The observed value was corrected for the background count and the "dead-time" of the instrument. A standard curve was plotted for each assay relating the number of counts per min. per ml. to various dilutions of the amount of I^{131} administered to the rats. From these data, a factor was obtained for the estimation of the percentage of the injected dose of I^{131} accumulated by each rat thyroid in 24 hr.

Results and Discussion

Influence of the Amount of Iodinated Casein in the Diet on the Thyroidal Uptake of I^{131}

Table I shows the effect of feeding iodinated casein at various levels in the food on the thyroidal uptake of I^{131}. The ingestion of the diet containing 0.005% iodinated casein caused almost maximum inhibition of the iodine

* *The basic diet contained 0.4% sodium chloride, 2.4% brewer's yeast, 1.5% alfalfa leaf meal, 3.7% crude casein, 12.0% flaxseed meal, 2.5% whole milk powder, 55.0% powdered corn meal, and 0.05% cod liver oil.*

† *"Protamone" was obtained from Cerophyl Laboratories Inc., Kansas City, Missouri. The biological properties of iodinated casein have been described by Reineke and Turner (14).*

TABLE I

INFLUENCE OF INGESTED IODINATED CASEIN ON THE THYROIDAL UPTAKE OF I^{131} IN THE 21-DAY-OLD RAT. THE THYROIDS WERE REMOVED 24 HR. AFTER THE INTRAPERITONEAL INJECTION OF APPROXIMATELY 2 MICROCURIES OF I^{131}

Number of rats	Average food consumption, gm./day/rat	Amount of iodinated casein in the diet, %	Thyroidal uptake of I^{131}, percentage of injected dose
10	5.7	0.000	26.79 ± 0.98%*
10	5.0	0.005	0.205 ± 0.015%
10	4.9	0.010	0.178 ± 0.014%
10	5.2	0.020	0.165 ± 0.020%

* *Standard error of the mean.*

accumulating activity of the rat thyroid. Although the retention of I^{131} was depressed still further by increasing the intake of iodinated casein, the differences observed were not significant.

Fig. 1 demonstrates that the subcutaneous injection of exogenous thyrotrophin at dose levels of 0.08 and 0.32 U.S.P. units stimulated the thyroids of rats fed iodinated casein to collect I^{131} in proportion to the dose given. The ability of these pretreated rats to differentiate between the amounts of thyrotrophin injected was dependent on the degree of inhibition of the thyroid gland. The slopes of the dosage–response lines were decreased as the amount of iodinated casein fed in the diet was increased.

These results are for the most part in accordance with those reported by Overbeek and co-workers (9). However, the percentage I^{131} uptake by the

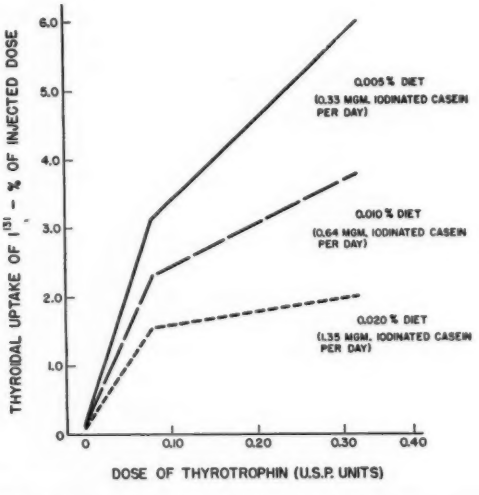

FIG. 1. Effect of dietary iodinated casein on the response of the weanling rat thyroid to exogenous thyrotrophin.

thyroid after treatment with iodinated casein was consistently lower in our experimental animals. This apparent disagreement between the results obtained by the two laboratories may be attributed to a difference in either the sensitivity of the rats used, or the biological activity of the particular brand of iodinated casein employed.

The Log Dose – Response Curve

Fig. 2 illustrates the relationship between the log dose of the thyrotrophin and the percentage of the injected dose of radioactive iodine retained by the thyroid of rats given iodinated casein. The response metameter plotted as a straight line over the dose range of 0.01 to 0.08 U.S.P. units of thyrotrophin when iodinated casein was fed at a level of 30 mgm. per kgm. of diet (0.003%). When the amount of iodinated casein in the food was increased to 50 mgm. per kgm. (0.005%) there was a shift in the position of the log dose – response curve so that the linear portion was between 0.02 and 0.16 U.S.P. units of thyrotrophin. The standard errors of the mean responses were comparatively uniform except at the higher dose levels where the amount of I^{131} collected by the thyroid approached a maximum value.

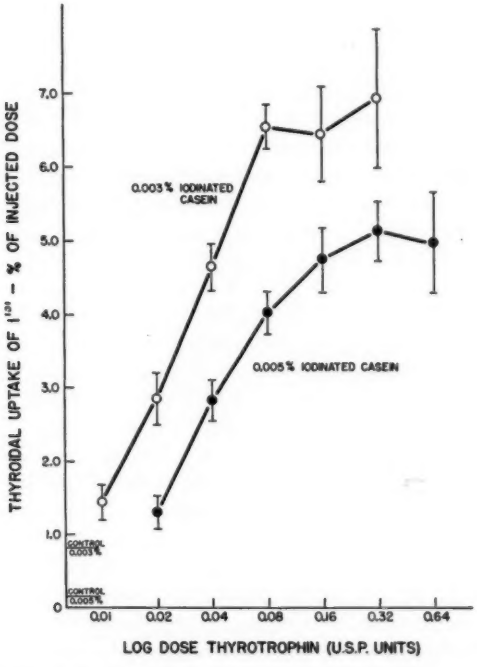

FIG. 2. Influence of dietary iodinated casein on the log dose – response curves for thyrotrophin; six rats per dose.

TABLE II

ANALYSIS OF THE DOSAGE – RESPONSE RELATION FROM THE DATA OBTAINED BY FEEDING 0.003% IODINATED CASEIN IN THE DIET. CORRECTION FOR VARIATIONS IN BODY WEIGHT BY COVARIANCE

Variance due to	Degrees of freedom	Sums of squares and products			Adjusted [y^2]		
		[w^2]	[wy]	[y^2]	Sum of squares	Mean or variance	Variance ratio, F
Doses							
(*a*) Linear	1	0	0	88.2710	88.2710	88.2710	217.042*
(*b*) Quadratic	1	1.500	0.755	0.3800	0.3681	0.3681	0.905
(*c*) Cubic	1	0.823	0.155	0.0288	0.0263	0.0263	0.065
Sex	1	150.000	− 24.300	3.8366	4.2340	4.2340	10.411*
Error	19	399.700	3.190	7.3464	7.3210	0.4067	
Total	23	552.033	− 20.200	99.8628			
Coefficients for adjusting [y^2]		0.0000637	0.01596	1			

* *Significant* ($P < 0.01$).
Note: Regression coefficient relating the body weight with thyroidal uptake of I^{131} = b_c = *0.007981.*
Slope "b" = 5.673.
Index of precision = s/b = *0.112.*

Table II shows an analysis of variance performed on the data illustrated in Fig. 2 obtained from rats pretreated with 0.003% iodinated casein and given doses of thyrotrophin ranging from 0.01 to 0.08 U.S.P. units. In addition, a correction was made for the variation in the body weight of the rats by covariance using the procedure described by Bliss and Marks (1). The regression coefficient or slope (b_c) of the straight line relating the body weight with the thyroidal uptake of I^{131} was very small, indicating that the hormonal response was not correlated significantly with the body weight. Consequently the correction for variations in body weight within dosage groups had no appreciable effect on the various terms in the analysis of variance table. The regression coefficient relating the thyroidal uptake of I^{131} with the log dose of thyrotrophin was highly significant. The other terms in the "between dosage groups" variation were not significant indicating that the regression line calculated by the method of least squares was linear over the range of 0.01 to 0.08 U.S.P. units of thyrotrophin. The variance ratio "F" for the between sexes term was also significant. Removal of this source of variation from the error lowered the standard deviation and therefore increased the precision of the assay.

Fig. 3 demonstrates that female rats provided, on the average, a higher response than male rats to graded doses of thyrotrophin. Since the males gained more weight than the females during the pretreatment period, a correction for this difference in body weight was made by estimating the dose of thyrotrophin on a per kgm. basis. The actual amount of the iodinated casein ingested per rat per day was 0.132 mgm. for females and 0.133 mgm. for

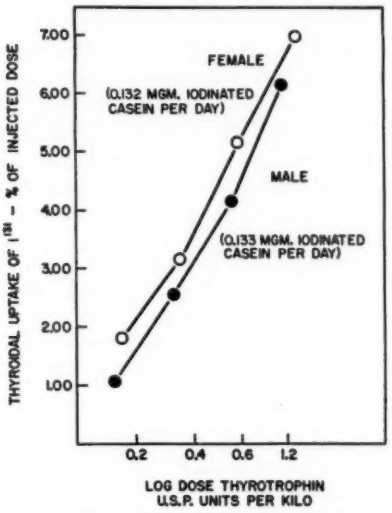

FIG. 3. Effect of sex on the log dose – response lines for thyrotrophin.

males. When the weight of the rats was considered, the average daily intake of iodinated casein was 2.26 mgm. per kgm. of female rat and 2.10 mgm. per kgm. of male rat. Therefore, a difference in the amount of iodinated casein ingested was not responsible for the increased responsiveness to thyrotrophin observed in female rats.

The Effect of the Dose of Radioactive Iodine on the Response to Thyrotrophin

Table III reveals that the percentage of the injected dose of I^{131} retained by the thyroid after 24 hr. at two dose levels of thyrotrophin was not related significantly to the dose of radioactive iodine injected. However, as shown

TABLE III

INFLUENCE OF THE AMOUNT OF I^{131} ADMINISTERED TO THE RAT ON THE THYROIDAL UPTAKE OF I^{131}

Dose of I^{131} injected, μc.	Thyroidal uptake of I^{131}, percentage of injected dose	
	0.03 U.S.P. units of TSH	0.06 U.S.P. units of TSH
2.0	4.20 ± 0.40%*	5.83 ± 0.58%*
2.1	2.76 ± 0.29	4.66 ± 0.28
3.5	4.10 ± 0.35	5.93 ± 0.44
3.7	3.82 ± 0.37	5.65 ± 0.37
4.2	3.79 ± 0.40	5.34 ± 0.39
17.3	4.30 ± 0.40	5.97 ± 0.98

* *Standard error of the mean.*

Note: Correlation coefficient between the dose of I^{131} and (1) the response at 0.03 U.S.P. units of TSH, $r = 0.449$ ($P > 0.3$); (2) the response at 0.06 U.S.P. units of TSH, $r = 0.432$ ($P > 0.4$).

in Table IV, lambda (λ) was found to be correlated significantly with the dose of I^{131} given ($r = 0.677$). Therefore the use of large amounts of the radioactive tracer tended to have a deleterious effect on the precision of the assay. Estimation of the correlation coefficients between the dose of I^{131} and the two components of the index of precision (s/b) indicated that the standard deviation of the assay data (s), but not the slope (b) of the log dose – response line, was related significantly to the I^{131} level injected into the rats.

TABLE IV

EFFECT OF THE AMOUNT OF I^{131} ADMINISTERED TO THE RAT ON THE PRECISION OF THE ASSAY

Dose of I^{131} injected, μc.	Standard deviation, s	Slope of the dosage response line, b	Index of precision
2.0	± 1.413	7.28	0.194
2.1	± 0.991	5.30	0.187
2.5	± 1.035	5.16	0.208
2.8	± 1.077	4.34	0.248
3.5	± 1.241	3.79	0.327
3.7	± 1.152	6.91	0.167
4.2	± 1.150	6.50	0.177
17.3	± 2.197	6.29	0.349

Note: Correlation coefficient between the dose of I^{131} and (1) the standard deviation (s), $r = 0.931$ ($P < 0.001$); (2) the slope of the dosage response line (b), $r = 0.191$ ($P > 0.6$); and (3) the index of precision (λ), $r = 0.677$ ($P < 0.05$).

The amount of radioactivity accumulated by the thyroid gland in 24 hr. was directly proportional to the quantity of the isotope administered. Consequently the increased variability in the response to thyrotrophin experienced at the higher dosage levels of I^{131} suggested that amounts of radioactive iodine had been injected into the rats which were harmful to the thyroid tissue.

Assay of a Commercial Preparation of Thyrotrophin

A thyrotrophin preparation, containing 10 U.S.P. units per vial according to a procedure based on the depletion of iodine in the thyroids of chicks (12, 17), was assayed in our laboratory using the thyroidal uptake of I^{131} in rats pretreated with iodinated casein as the response to the hormone. The weighted mean potency obtained by combining the results of two assays was 10.1 U.S.P. units per vial with confidence limits ($P = 0.05$) of 7.8 to 13.1 U.S.P. units per vial. Therefore the thyrotrophic activity, measured by the retention of iodine by the thyroid of rats, did not differ significantly from that determined by the depletion of iodine in the thyroids of chicks.

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THE EFFECT OF ATROPINE SULPHATE ON BLOOD AND URINE ELECTROLYTES IN MAN[1]

By E. H. Walker, J. M. Parker, and John Hunter

Abstract

Atropine sulphate (1 or 2 mgm.) was injected intramuscularly into human volunteers. Concentrations of sodium and potassium in the blood and urine were followed for three hours after the drug had been administered. A decreased packed cell volume was found, suggesting a transfer of interstitial fluid into the blood. No important change in the blood or urine sodium and potassium was shown in these subjects under the conditions of the experiment (74° F., 25% relative humidity). No harmful effects were noted in the volunteers after they received the atropine sulphate.

Introduction

It is well known that large doses of atropine produce a rise in body temperature (5). Goldberg, Ell, and Walton (4) have shown that artificially induced hyperthermia causes an elevation of serum potassium. Voloskov (10) has reported an increase of potassium in the blood of rabbits after large doses of atropine. The present work was undertaken to determine whether measurable electrolyte changes in the blood and urine of human subjects follow moderate doses of atropine sulphate.

Method

A 1 mgm. dose of atropine sulphate was injected intramuscularly into each of 11 volunteers. Thirteen volunteers later received a 2 mgm. dose. Studies started at 9.00 a.m. The subjects were sedentary in an environment of 74° F., 25% relative humidity. During the course of the experimental period, fluid intake was restricted. In the group receiving the 1 mgm. dose and in eight of those receiving the 2 mgm. dose, there was no restriction of previous fluid intake or diet. The five remaining subjects who received 2 mgm. had no food or water for 12 hr. previous to drug administration. One-half hour before drug administration, they ingested 200 ml. of water.

Blood pressures, pulse rates, and oral body temperatures were noted. Samples of blood and urine were collected at zero, one-half, one, two, and three hour intervals after drug administration. Packed cell volume determinations were made on each sample of blood. Sodium and potassium measurements were carried out on plasma, whole laked blood, and urine. On several days, when no drug was administered, urine was collected at hourly intervals and control values for sodium and potassium excretions were determined for the breakfast and non-breakfast groups.

A Cambridge continuous blood pressure indicator was used to measure blood pressure and pulse rates. Packed cell volume was estimated by means

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of the hematocrit. The blood was placed in a 10 cm. tube and centrifuged at 3000 r.p.m. for 60 min. The column of cells was read to the top of the white cell layer, and this value was adjusted to the height of the red cell column by subtracting 1 mm. (6). A correction for trapped plasma was made by multiplying the value for the cell volume by a factor of 0.95 (1). A Baird flame photometer was used to measure sodium and potassium by the direct method.

The results were examined statistically using Student's '*t*' method of analysis for paired variables.

Results

A summary of the results may be seen in Table I.

TABLE I

SUMMARY

	1 mgm. atropine sulphate (with breakfast)	2 mgm. atropine sulphate (with breakfast)	2 mgm. atropine sulphate (without breakfast)
Packed cell volume	Decreased*	Decreased	Decreased*
Whole blood sodium	No change	Slight decrease*	Slight decrease*
Whole blood potassium	No change	No change	No change
Plasma sodium	No change	No change	No change
Plasma potassium	Slight increase*	No change	No change
Urinary sodium	Slight increase	Slight increase	Slight increase
Urinary potassium	No change	No change	No change
Urinary volume	No change	No change	No change
Heart rate	Increased*	Increased*	Increased*
Systolic blood pressure	Decreased*	Decreased*	Decreased*
Diastolic blood pressure	Increased*	Increased*	Increased*
Pulse pressure	Decreased*	Decreased*	Decreased*

* *Significance at 5% level. For details see text.*

The results obtained with the 1 mgm. dose of atropine sulphate are given in Table II and Table III. There was a small decrease in the average packed cell volume from a normal value of 47.6 ± 1.00% to 45.7 ± 1.02% three hours after the drug was given. No change was evident in the whole blood sodium or potassium. There was no apparent change in the concentration of sodium in the plasma. There was a slight increase in the plasma potassium concentration averaged over the three hour period after drug administration from a control value of 20.2 ± 0.90 to a value of 22.6 ± 0.60 mgm./100 ml. The latter increase was statistically significant ($t = 2.60$, $p < 0.05$). In seven of ten subjects, the total amount of sodium excreted in the urine during

TABLE II

THE EFFECT OF ATROPINE SULPHATE ON PACKED CELL VOLUME AND CONCENTRATION OF SODIUM AND POTASSIUM IN THE PLASMA

Time after drug, hr.	1 mgm. atropine sulphate (with breakfast)			2 mgm. atropine sulphate (with breakfast)			2 mgm. atropine sulphate (without breakfast)		
	Packed cell volume, % cells	Plasma		Packed cell volume, % cells	Plasma		Packed cell volume, % cells	Plasma	
		Sodium, mgm./100 ml.	Potassium, mgm./100 ml.		Sodium, mgm./100 ml.	Potassium, mgm./100 ml.		Sodium, mgm./100 ml.	Potassium, mgm./100 ml.
0	47.6	363.2	20.3(N =10)	47.8	362.8	21.0(N =7)	49.9	324.1	19.4
½	46.5	363.3	22.0	47.6	362.8	20.9(N =7)	46.8	324.5	20.1
1	46.3	368.6	22.7	46.8	359.8	21.5(N =7)	48.5	329.8(N =4)	19.1(N =4)
2	46.0	363.5	21.7	47.0	354.9	20.5(N =7)	48.1	333.5	20.4
3	45.7	370.1	23.1(N =10)	47.9(N =6)	362.0	20.9(N =7)	48.6	336.7(N =4)	20.7(N =4)
	N = 11 except where specifically noted			*N = 8 except where specifically noted*			*N = 5 except where specifically noted*		

the three hour post drug period was greater than for similar three hour control periods. No apparent effect of atropine on potassium excretion or on urine volume was noted.

Atropine sulphate (2 mgm.) was given to two groups of subjects. One group of eight had breakfast prior to drug injection, and the second group of five went without food or water from 9.00 p.m. the previous night to one-half hour before drug administration. One-half hour before drug injection, these subjects ingested 200 ml. of water. Control urine samples were obtained from the two groups.

In the first group, the packed cell volume decreased in five of the eight subjects. The decrease was of a similar magnitude to that obtained with the 1 mgm. dose, but the onset of the drug effect was much earlier with the 2 mgm. dose than with the 1 mgm. dose. In six of eight subjects there was a small decrease in the sodium concentration in whole blood. The potassium concentration in the whole blood was unchanged. No effect of atropine on sodium or potassium in plasma was noted in these experiments. There was an indicated increase of sodium excreted in the urine over the three hour period after drug administration in six of eight subjects, but potassium excretion and urine volume were not altered. The results obtained are given in Tables II and III.

TABLE III

THE EFFECT OF ATROPINE SULPHATE (1 OR 2 MGM.) ON URINE VOLUME AND URINARY EXCRETION OF SODIUM AND POTASSIUM

Time after drug, hr.	Average urinary excretion					
	Sodium (mgm.)		Potassium (mgm.)		Volume (ml.)	
	Drug	Control	Drug	Control	Drug	Control
1 mgm. atropine sulphate (with breakfast)—10 subjects						
1	289.9	171.4	224.9	165.0	72	53
2	255.1	192.5	210.2	201.1	76	58
3	215.5	180.9	218.1	206.8	60	51
Total	760.5	544.8	653.2	572.9	208	162
2 mgm. atropine sulphate (with breakfast)—8 subjects						
1	281.8	183.5	203.6	174.9	53	59
2	202.3	205.8	162.5	182.1	62	56
3	236.3	198.9	235.4	217.7	73	55
Total	720.4	588.2	601.5	574.7	188	170
2 mgm. atropine sulphate (without breakfast)—5 subjects						
1	184.2	126.5	152.6	163.5	90	56
2	128.3	130.6	113.7	169.8	90	66
3	118.2	75.1	100.9	117.8	93	30
Total	430.7	332.2	367.2	451.1	273	152

TABLE IV

THE EFFECT OF ATROPINE SULPHATE ON BLOOD PRESSURE AND HEART RATE

Time after drug, hr.	Systolic blood pressure, mm. Hg			Diastolic blood pressure, mm. Hg			Pulse pressure, mm. Hg			Heart rate, beats/min.		
	I	II	III	I	II	III	I	II	III	I	II	III
0	119	116	121	77	77	79	41	38	42	71	89	71
½	115(N=10)	116	122	80	82(N=9)	82	33(N=10)	34(N=7)	41	82(N=10)	107	100
1	113	118	117	83	89	85	29	28	31	83	107	103
2	112	111	118	82	84	85	30	27	33	75(N=10)	90	71
3	112(N=8)	115	116	79(N=8)	84	84	32	30	31	73	77	78(N=4)

Group I: 1 mgm. atropine sulphate with breakfast, N=11.
Group II: 2 mgm. atropine sulphate with breakfast, N=8.
Group III: 2 mgm. atropine sulphate without breakfast, N=5.

In the second group receiving 2 mgm., there was a decrease in the packed cell volume, in the first half hour after drug administration, from a normal of 49.9 ± 1.4% to 46.8 ± 1.6%. In four of the five subjects, there was a small decrease in the concentration of sodium in whole blood in the first two hours after the drug was given. No effect on whole blood potassium was observed. Plasma concentrations of sodium and potassium were not altered. In four of the five subjects, there was a slight increase in sodium excreted during the three hour period after drug administration. Neither urine potassium nor urine volume was affected in these experiments. The results obtained are given in Tables II and III.

No obvious difference between the two groups receiving 2 mgm. atropine sulphate was noticed, except for plasma sodium concentrations and sodium excreted in the urine, which were higher in the group which had breakfasted.

The effects of atropine sulphate on blood pressure and heart rate are shown in Table IV. The heart rate was accelerated in all subjects. The larger dose produced a greater acceleration over a longer period of time. The decrease in pulse pressure was of a similar order with both the 1 and 2 mgm. doses and was significant at the 5% level, one hour after the drug was given [$t = 2.8$ (1 mgm.) and $t = 2.2$ (2 mgm.)].

At these doses of atropine, the oral body temperatures of the subjects at room temperature (approximately 74° F. and 25% relative humidity) did not vary by more than a degree from the normal.

Discussion

Since atropine sulphate in 1 and 2 mgm. doses does not cause any apparent change in the concentration of sodium in the plasma, the decreased packed cell volume suggests the entrance of interstitial fluid into the blood, on the assumption that the sodium concentration of interstitial fluid approximates that of plasma. This does not exclude possible entrance of fluid from the gastrointestinal tract (2, 7).

Sirasaka (8) and Traverso and Gavazzeni (9) have reported an increased blood volume in dogs after atropine was administered. Fliederbaum (3) reported that the amount of water circulating in the blood plasma of dogs was increased by atropine. The doses used were not stated. The work of these authors supports the finding of this investigation concerning the decreased packed cell volumes in human subjects.

Significant differences in plasma sodium concentration and total urine sodium output were observed between the breakfast and non-breakfast groups. The differences between these groups were greater than any difference in sodium excreted in the urine or concentration of sodium in the blood caused by atropine within each group.

The sodium and potassium concentrations in the blood or urine of the subjects used under these experimental conditions showed little change of importance after atropine administration.

Atropine sulphate caused an elevated heart rate. A decreased pulse pressure was evident, due to both an increased diastolic pressure and a decreased systolic pressure.

Conclusions

1. A slight decrease in the packed cell volume was observed after 1 or 2 mgm. atropine sulphate had been injected intramuscularly into human volunteers.

2. No alteration in whole blood sodium or potassium was noted with the 1 mgm. dose. There was a small but statistically significant reduction in whole blood sodium with the 2 mgm. dose. Potassium concentration in whole blood was unchanged by the 2 mgm. dose.

3. Concentrations of sodium and potassium in the plasma varied within normal limits after 1 or 2 mgm. of atropine sulphate was given.

4. There was an indicated increase in the sodium excreted in the urine in the three hour interval after drug administration. No changes in potassium excretion or urine volume were observed.

5. Pulse pressure was decreased approximately 10 mm. Hg. This decrease was significant at the 5% level.

6. Heart rate was accelerated, the 2 mgm. dose producing a greater acceleration over a longer period of time.

7. Oral body temperature was not greatly affected by the doses used.

8. No harmful effects were observed in any of the subjects participating in this study.

9. The data suggest that there may be a transfer of interstitial fluid into the blood with administration of atropine, on the assumption that the sodium and potassium concentrations of the interstitial fluid approximate that of blood.

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THE SEPARATION OF CALF THYMUS NUCLEIC ACID AND HISTONE BY ALCOHOL–SALT PRECIPITATION[1]

By L. B. Smillie[2], A. M. Marko[3], and G. C. Butler

Abstract

Extraction of the histone of thymonucleoprotein with alcohol and salt has been studied with varying concentrations of alcohol, sodium chloride, and nucleoprotein and with varying pH and temperature. The addition of 0.5–1.0 volumes of ethanol to a solution of nucleoprotein (approximately 1 mgm. N/ml.) in 3 *M* sodium chloride has been found to effect an almost quantitative separation of desoxyribonucleic acid and protein. It has proved feasible to prepare concentrated aqueous solutions of the extracted protein by successive dialysis against strong salt solutions at −10° C. and against distilled water at 5° C. followed by pervaporation at 5° C. The properties of the isolated nucleate have indicated a highly polymerized product.

Introduction

Although acceptable methods for the isolation of desoxyribonucleate (DNA) have been in use for a number of years (8, 13, 14) a completely satisfactory procedure for the isolation of the protein associated with the nucleate has not been developed. Hydrochloric and sulphuric acids have been used by many workers (6, 11, 15) for the extraction of histone from desoxyribonucleoprotein, but at the initiation of this work, the acid extraction method appeared to suffer from both its non-quantitative yield and its harshness.

S.S. Cohen described the separation of histone and nucleic acid by treatment with alcohol and salt (2). Other workers have also recently reported the action of alcohol in extracting histone and in precipitating DNA (1, 4, 7) but no systematic study of the variables involved has been made. For this reason the effects of varying concentrations of sodium chloride, ethanol, and thymonucleoprotein (TNP) and of varying pH and temperature on the extent of protein extraction were studied in detail. These and other studies of isolation and concentration have made possible the quantitative separation and isolation of both the components of calf TNP.

Experimental

General Methods

Analyses were made for nitrogen according to Ma and Zuazaga (12) and for phosphorus by King's (10) method for total phosphorus.

TNP was prepared by the modified method of Mirsky and Pollister (14) and is referred to in subsequent experiments as "crude TNP". The TNP in a solution of 1 *M* sodium chloride was further purified by pouring the viscous solution into six volumes of 0.01 *M* sodium citrate and stirring the mixture occasionally over a period of five hours. After decantation of the supernatant solution, the precipitate was redissolved in 1 *M* sodium chloride

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containing 0.01 *M* sodium citrate. The precipitation and resolution were repeated twice more to yield a product which was very opalescent and contained much insoluble material. By adjusting the concentration to 1 to 1.5 mgm. of nitrogen per ml. and centrifuging the mixture at 20,000 r.p.m. for two hours the insoluble material could be sedimented leaving a TNP solution only slightly opalescent. The nucleoprotein in such solutions has been designated "purified TNP".

In the experiments to be described below the percentage extraction of protein nitrogen has been calculated assuming a nitrogen: phosphorus ratio of 1.68 for pure calf thymus DNA. This value was calculated from mononucleotide analyses of DNA made in this laboratory (9).

Alcohol–Salt Extraction of Histone

(1) Effect of Sodium Chloride Concentration

To samples of "crude TNP" solution in 50 ml. beakers were added varying amounts of solid sodium chloride to establish sodium chloride concentrations varying from 1 *M* to 6 *M*. After solution of the salt the beakers were allowed to stand for 15 hr. at 5° C. To each beaker was then added an equal volume of 95% ethanol, and the mixture was stirred until the fibrous precipitate was wound tightly around the stirring rod. Each precipitate was removed and dispersed in a Potter–Elvehjem homogenizer with 5 ml. of ethanol – sodium chloride solutions, corresponding to the final concentrations used in the initial extractions. The resulting mixture was centrifuged for 15 min. at 2000 r.p.m., the supernatant solution decanted and the residue washed twice more by dispersal, centrifugation, and decantation. The combined supernatants after filtration through Whatman No. 1 filter paper were analyzed for nitrogen and phosphorus. The results of these analyses expressed as % of protein nitrogen

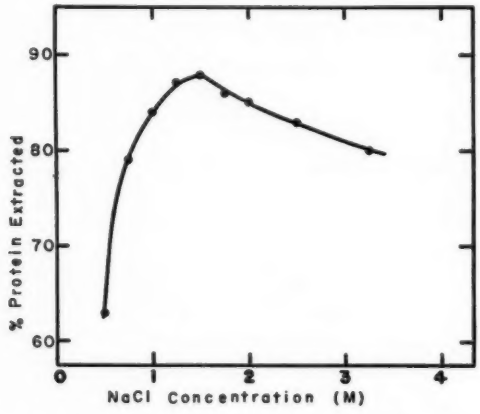

FIG. 1. Effect of sodium chloride concentration on extraction of histone with ethanol and sodium chloride. The final mixtures contained "crude TNP" (0.75 mgm. N per ml., N: P = 4.67), ethanol (50%), and sodium chloride; at 25° C. pH 6.8.

extracted are presented in Fig. 1. Maximum extraction was achieved with a final sodium chloride concentration of 1.5 *M*. The smaller extractions at lower sodium chloride concentrations are probably due to incomplete dissociation of the nucleoprotein complex. At higher concentrations of sodium chloride there may be a salting out of the protein from the supernatant. Crampton, Lipshitz, and Chargaff (4) have also found that as the salt concentration is increased a greater extraction of protein is achieved. Their final sodium chloride concentration, however, did not exceed 0.86 *M* at which concentration they achieved 90 to 95% extraction of the protein.

(*2*) *Effect of Ethanol Concentration*

To a series of 50 ml. beakers containing 5 ml. of "crude TNP" in 5 *M* sodium chloride, 20 ml. of varying concentrations of aqueous ethanol was added. The methods of removing the fibrous precipitate and of grinding and washing it were similar to those described above. The results of the nitrogen and phosphorus analyses are shown in Fig. 2. With 30% alcohol the DNA failed to precipitate when the ethanol was added rapidly and a figure for this ethanol concentration is not shown. In later work, however, it was found that slow addition of the alcohol, accompanied by constant stirring, to a final concentration of 30% precipitated the DNA. From these results it is evident that the extraction of nitrogen remained constant with final ethanol concentrations varying from 30 to 60%. At alcohol concentrations greater than 60% the yield was smaller.

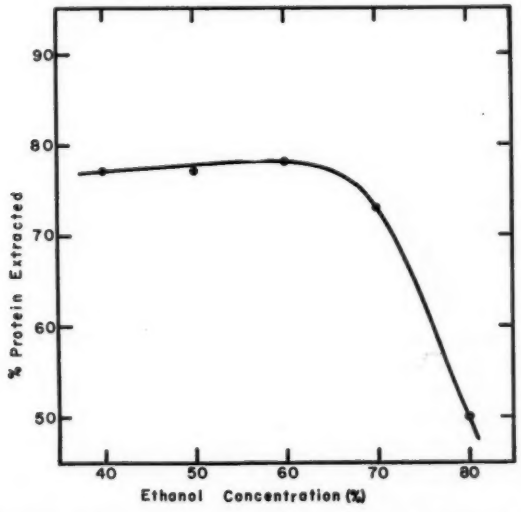

FIG. 2. Effect of ethanol concentration on extraction of histone with ethanol and sodium chloride. The final mixtures contained "crude TNP" (1.04 mgm. N per ml.), sodium chloride (1 *M*), and ethanol; at 25° C. and pH 6.8.

(*3*) *Effect of pH*

Forty milliliter portions of "crude TNP" in 3 *M* sodium chloride solution were pipetted into 100 ml. beakers and the pH was adjusted with 0.1 *N* hydrochloric acid or sodium hydroxide. After the pH was adjusted the thymonucleoprotein solutions were transferred to 50 ml. volumetric flasks by washing in with 3 *M* sodium chloride solution and the pH values were redetermined. Then 10 ml. aliquots of the thymonucleoprotein solutions were dispensed into a series of 50 ml. beakers and the solutions were mixed with 10 ml. of absolute alcohol as described previously.

The results of the nitrogen and phosphorus analyses are recorded in Fig. 3. The maximum extraction of protein nitrogen occurred at pH 7.5.

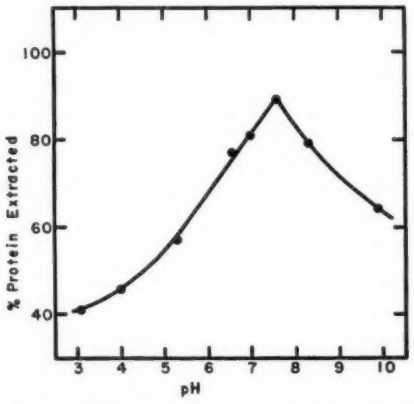

FIG. 3. Effect of pH on extraction of histone with ethanol and sodium chloride. The final mixtures contained "crude TNP" (0.585 mgm. N per ml., N: P = 4.89), sodium chloride (1.5 *M*), and ethanol (50%); at 25° C.

(*4*) *Effect of TNP Concentration*

Solutions with varying concentrations of "crude TNP" were prepared in 3 *M* sodium chloride. Equal samples of the different solutions were obtained by weighing 10 gm. samples in 50 ml. beakers. These solutions were then treated with an equal volume of absolute alcohol in the usual way. The results of nitrogen and phosphorus analyses are presented in Fig. 4.

The % nitrogen extracted decreases as the nucleoprotein concentration increases. This may be due to two factors; first, the nucleoprotein is less dissociated at high concentrations and second, the protein may have a limited solubility in the ethanol–salt solution.

(*5*) *Effect of Temperature*

Samples of "crude TNP" in 3 *M* sodium chloride were brought to temperatures varying from −30° to +25° C. and extracted in the usual way with alcohol at the same temperature. The results of nitrogen and phosphorus

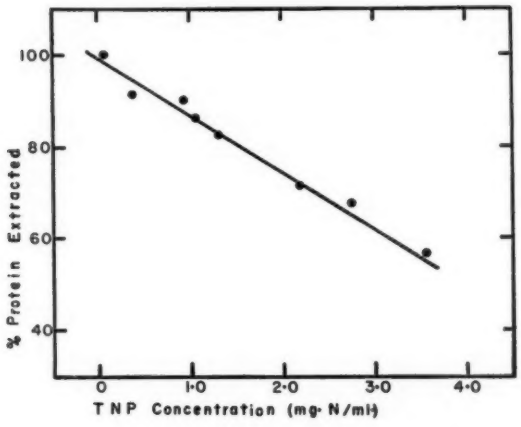

FIG. 4. Effect of TNP concentration on extraction of histone with ethanol and sodium chloride. The final mixtures contained sodium chloride (1.5 *M*) and ethanol (50%); at 25° C. and pH 6.8.

analyses indicated that the percentage protein extracted was about the same for all temperatures. Nevertheless, the use of low temperatures is advisable when proteins are being treated with alcohol since Cohn and Edsall (3, 5) have shown that the denaturing effects of alcohol are minimized at temperatures below −5° C.

(*6*) *Finally Adopted Procedure*

The above experiments formed the basis of the following general method of extracting the protein: A solution of TNP in 3 *M* sodium chloride, adjusted to a concentration of about 1 mgm. of nitrogen per ml., is cooled to −10° C. Absolute alcohol is cooled to the same temperature as the nucleoprotein solution and added slowly with stirring to a final ethanol concentration of 30–50%. The temperature is not allowed to rise above −8° C. The fibrous precipitate is wound around a stirring rod, compressed against the side of the container, drained, and removed. No further washing or grinding of the precipitate has been found necessary for the quantitative separation of the two components.

(*7*) *"Purified TNP"*

The use of the general method described above with "crude TNP" gave extractions of only 70 to 90% of the calculated protein content of the TNP. These apparently low yields were due to the insoluble material present in these crude preparations. When the method was applied to "purified TNP" the separation of TNP into DNA and protein was found to be almost quantitative. The results of four such experiments are shown in Table I.

TABLE I

EXTRACTION OF "PURIFIED TNP" WITH 33% ETHANOL AND 2 *M* SALT AT −8° TO −10° C.

Expt. No.	N: P ratio of purified nucleoprotein	N: P ratio of alcohol–salt extract	N: P ratio of alcohol–salt precipitate	Calculated % extraction of protein
1	3.70	251	1.72	98.2
2	3.80	131	1.70	99.2
3	3.51	297	1.69	99.3
4	3.80	238	1.69	99.6

Isolation of Protein

The supernatant solutions obtained in the extraction procedure described above contain 25 to 40 mgm. of nitrogen per ml. or about 0.2% protein. For physical–chemical characterization of the protein, the alcohol and salt must be removed and the concentration increased to 0.5–1.0%.

(*1*) *Precipitation Procedures*

Attempts to precipitate the protein by the addition of neutral salts were found to be impractical because the majority of salts such as sulphates, phosphates, citrates, and carbonates are not sufficiently soluble in 30–50% ethanol. Ether was found to be immiscible with the aqueous alcohol–salt solutions. Acetone was used but it precipitated the sodium chloride in addition to the protein.

(*2*) *Dialysis Against Distilled Water*

When the alcohol–salt extract was dialyzed against distilled water at 5° C., a flocculent precipitate appeared, comprising, in a number of preparations, from 20 to 55% of the extracted nitrogen (Table II). The precipitated protein was not soluble in salt solutions, dilute acid, or alkali. Butler *et al.* (1) have also obtained an insoluble fraction amounting to about one third of the extracted protein. This fraction was likewise insoluble in mild reagents. Grégoire and Lémozin (7), however, claim that this insoluble fraction goes into solution by prolonged dialysis against distilled water. Such has not been our experience.

(*3*) *Removal of Alcohol by Lyophilization*

Since the insoluble fraction possibly arose from alcohol denaturation of the protein, the removal of the alcohol while the extract was maintained at a low temperature was attempted by freeze-drying. Considerable bubbling of the mixture occurred during this procedure since the rate of evaporation was not sufficient to keep the alcohol–salt solution frozen. When dry, the protein was taken up in a small amount of water and dialyzed first against running tap water and finally against frequent changes of distilled water. The insoluble protein was separated by centrifugation at 2000 r.p.m. The results of three experiments are shown in Table II. No reduction in the amount of insoluble protein was achieved.

(4) Removal of Alcohol by Dialysis at −10° C.

Since 3.0 *M* sodium acetate and 3.75 *M* sodium chloride do not freeze at −10° C., the alcohol can be removed from the extracts by dialysis against these solutions. Extracts prepared at −8° to −10° C. were dialyzed for 8 to 12 hr. against one or the other of these two salt solutions at −10° to −15° C., then against running tap water at 5° C. for 12 hr., and finally against several changes of distilled water at 5° C. The insoluble protein was separated by centrifugation at 2000 r.p.m. The results of five experiments are presented in Table II.

TABLE II

PER CENT OF PROTEIN RENDERED INSOLUBLE DURING REMOVAL OF ALCOHOL

Dialysis against distilled water at 5° C.	Freeze-drying	Dialysis at −10° C. against	
		Sodium acetate	Sodium chloride
24.5	18.3	6.5	4.4
39.0	37.1	1.2	6.0
33.0	11.4	—	6.1
22.4	—	—	—
54.3	—	—	—

It can be seen that the best results were obtained by dialysis against a concentrated salt solution at −10° C. The insoluble fraction was separated from the soluble protein in the above experiments by centrifugation at 2000 r.p.m. The supernatant after this treatment was still opalescent and contained more insoluble protein. Thus the results reported in Table II are only relative and the insoluble fraction probably comprised in every case a slightly larger percentage than the figures indicate.

In a number of further experiments the alcohol was removed by dialysis at −10° C., followed by dialysis at 5° C. against running tap water and against distilled water in turn. The insoluble fraction was separated by centrifugation at 5000 r.p.m. for 15 to 30 min. which left the supernatant entirely free of opalescent material. Analyses indicated that a total of 12 to 22% of the protein had been rendered insoluble. No further reduction of the amount of protein rendered insoluble by the alcohol has yet been achieved.

During dialysis, in addition to the protein rendered insoluble, there occurs a loss of nitrogen through the dialysis membrane. Varying from about 29% in one or two experiments to only 2.5 in another, the average loss in 20 experiments was 17.2% of the total extracted nitrogen. This loss may be due to one of two factors; firstly, low molecular weight, non-protein, nitrogenous substances may make up a portion of the alcohol–salt extract; secondly,

the protein or a fraction of it may be of sufficiently small molecular size to pass through the dialysis membrane. In the latter case the amount of nitrogen lost would depend upon the length of time of dialysis and the tension upon the dialysis bag. The large variation in the nitrogen loss may be accounted for in this way.

(5) *Concentration of the Protein*

The most successful method of concentration has been by pervaporation at 1° to 2° C. Only a small fraction of the protein (4 to 8%) is rendered insoluble during this process and if the humidity of the cold room is low, several liters of solution can be concentrated to a few hundred milliliters in two to three days. Other methods of concentration such as freeze-drying and ultrafiltration were not as successful. The former rendered a portion of the material insoluble while ultrafiltration was technically difficult owing to the passage of the small protein molecules through the filtration membrane.

Isolation and Properties of Desoxyribonucleate

The fibrous DNA, precipitated by alcohol from salt solutions of purified nucleoprotein, is removed and washed with alcohol and ether as described previously by Marko and Butler (13). The dried nucleate is readily soluble in water. Properties of solutions of the nucleate are similar to those of nucleate prepared in this laboratory with sodium dodecyl sulphate (13). The $\eta_{sp}(P)$* in 0.02 *M* sodium chloride for one sample was 35.3 and in water was 64.5. The extinction coefficient $\epsilon(P)$† at 2300 Å was 2980 and at 2600 Å was 6620. Nitrogen: phosphorus ratios for four preparations may be seen in Table I.

Discussion

Salt probably functions in two ways in the separation of histone and nucleate by alcohol–salt precipitation. Firstly, it produces dissociation of the nucleic acid – protein complex. This effect of sodium chloride on the dissociation of nucleic acid and protein has been demonstrated previously (4, 13, 14). From the results presented in this paper it appears that this dissociation is complete in 1.5 *M* sodium chloride. Indeed, it may well be that the solubility of the nucleoprotein in salt solutions depends entirely on this dissociation. Secondly, the sodium chloride has a salting-in action on the protein in solutions of alcohol sufficiently concentrated to render the sodium desoxyribonucleate insoluble. Edsall (5) has described a similar behavior for a number of proteins.

Butler *et al.* (1) have reported the extraction of histone from TNP with alcohol and salt leaving a nucleate residue with a nitrogen: phosphorus ratio of 1.9. They attribute the non-quantitative yield to pentosenucleic acid

* $\dfrac{\textit{Specific viscosity}}{\textit{Phosphorus concentration (gm. per liter)}}$

† $\dfrac{\textit{Optical density}}{\textit{Phosphorus concentration (moles per liter)}}$

present in the preparations in concentrations up to 8%. Our TNP preparations have contained from 2 to 6% pentosenucleic acid and in our experience apparent non-quantitative yields have been due to the presence of insoluble protein material suspended in the very viscous nucleoprotein solutions. When this contamination has been removed by high-speed centrifugation, separation of protein and DNA has been almost complete.

As a method for the preparation of nucleic acid the alcohol–salt method has certain advantages. For small scale preparations the method is relatively simple and rapid, and the properties of the DNA are indicative of a pure and highly polymerized product. For large preparations, however, the high-speed centrifugation required for the preparation of "pure TNP" is a laborious and time-consuming step. If the nucleic acid is to be used for the preparation of nucleotides the centrifugation may be omitted and the insoluble material removed by filtration after depolymerization of the nucleic acid.

Properties of the extracted protein are now being studied. Results obtained up to the present indicate that the protein has been altered from its native state by the extraction conditions.

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A STUDY OF THE AUTONOMIC MANIFESTATIONS SEEN IN ACUTE ALDRIN AND DIELDRIN POISONING[1]

By C. W. Gowdey and G. W. Stavraky

Abstract

The peripheral parasympathomimetic action of aldrin was investigated in vagotomized and adrenalectomized cats under chloralose and urethane anesthesia. Under these experimental conditions aldrin caused slowing of the heart, potentiated the effects of electrical stimulation of the vagus nerve, and augmented the secretory effect of the chorda tympani on the decentralized submaxillary salivary gland. Blood withdrawn five minutes after intravenous injections of aldrin into cats showed a reduced rate of destruction of added acetylcholine when tested on the frog's rectus abdominis muscle. In spite of a marked central action, dieldrin exerted none of these peripheral effects.

Introduction

The structural formulae for aldrin and dieldrin are shown below in Fig. 1.

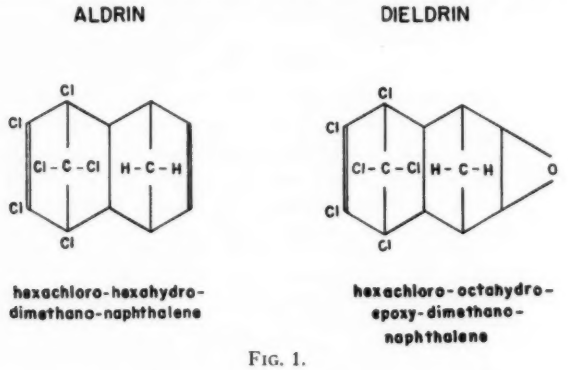

Fig. 1.

In a previous study of the mode of action of aldrin in the mammal (10) it was found that shortly after aldrin administration a previously subthreshold stimulation of the vagus nerve produced marked cardiac slowing and a fall in blood pressure; aldrin also potentiated and prolonged the effects produced by a given dose of acetylcholine. In addition, it augmented the secretory and vasodilator responses of the submaxillary gland to stimulation of the chorda tympani and to injections of acetylcholine. Intestinal motility was increased by small doses of aldrin but decreased by large doses. Miosis also occurred. Atropine annulled the parasympathomimetic action of aldrin. These effects of aldrin were qualitatively similar to those of physostigmine, and could be explained by assuming that aldrin acts at postganglionic parasympathetic sites.

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It was also demonstrated (10) that aldrin potentiated certain effects of acetylcholine not related to the autonomic nervous system. Thus it increased the excitability of spinal neurones to intra-arterial injections of acetylcholine, produced muscle twitches, potentiated the crossed extensor reflex, and augmented transmission across the neuromuscular junction. Repeated injections of aldrin eventually depressed the reflexes and blocked the neuromuscular junction.

It is true that, unlike physostigmine, aldrin had no effect when applied locally to the eye, or to isolated intestinal or uterine strips *in vitro*, presumably because of its low aqueous solubility. The main difficulty at the time of this study was to explain the mechanism of action of aldrin. In spite of the fact that it produced all of the above-mentioned effects, Smallman (13) found that aldrin had no anticholinesterase activity in insects, and Crevier, Monkman, and Ball (7) reported an increase in serum esterase activity in rats after chronic poisoning with aldrin. In a recent paper, however, Crevier, Ball, and Kay (6) have reported that in the early stages of aldrin poisoning a significant fall in serum esterase preceded the rise.

Dieldrin, which is closely related chemically to aldrin (Fig. 1), was found to have a much more pronounced action on the central nervous system than on peripheral tissues (11). Dieldrin also caused convulsive twitches, increased the reflex excitability of the spinal cord, and potentiated its responses to injected acetylcholine. On the other hand, dieldrin did not affect the isolated submaxillary salivary gland, and although it produced cardiac slowing and increased the responses of the heart to acetylcholine, this effect disappeared when the vagi were sectioned. Similar effects on intestinal motility were observed, the augmentation disappearing on vagal section.

The present investigation was designed to define further the mechanism of action of these two chlorinated-hydrocarbon insecticides in the mammal under conditions of acute poisoning.

Methods

The experiments on the circulatory system and on the salivary gland were performed on cats anesthetized with a mixture of chloralose (50 mgm. per kgm.) and urethane (500 mgm. per kgm.). Cannulae were tied into the trachea, one common carotid artery, femoral artery, and femoral vein. The submaxillary salivary duct was cannulated and connected to an electronic drop recorder; the submaxillary vein was isolated, and the blood flow through the gland was recorded. The chorda tympani and cervical sympathetic trunk were severed in the neck and the peripheral ends stimulated with a bipolar electrode connected to a Harvard Inductorium. In 10 cats both vagus nerves were severed in the neck, and in five of these both adrenal glands were removed. The distal end of the right vagus was stimulated electrically, and the threshold for cardiac slowing determined before and after aldrin administration. The arterial blood pressure and pulse rate were studied by means of manometric recordings.

Aldrin (technical Octalene, Compound 118) and dieldrin (Compound 497) were dissolved in 95% ethanol, and the solutions injected in volumes of 1 ml. or less. No effects were observed with equal volumes of ethanol alone.

Because of the low aqueous solubility of these compounds the usual pharmacological techniques for measuring anticholinesterase activity could not be used. It was therefore decided to inject aldrin or dieldrin intravenously into cats and to measure the *in vitro* rate of destruction of acetylcholine added to blood samples withdrawn five minutes later. For this purpose the frog rectus method was used.

The frog was pithed and both rectus abdominis muscles removed together. The muscle was placed in an isolated organ bath (6 ml. capacity) filled with frog Ringer's solution; the pubic end was fixed and the sternal connected to a light gimbal lever by a silk thread. The bath was aerated continuously at room temperature throughout the experiment. After an initial period of equilibration with a 2 gm. weight on the lever, the test solutions were applied at five-minute intervals and left in contact with the muscle for 90 sec. Between applications the muscle was rinsed twice with, and bathed in, Ringer's solution, and the lever weighted with 2 gm.

The test samples (10 ml.) of cat's blood were removed from the femoral artery into 0.5 ml. of heparin solution (5 mgm./ml.) before, and 5.0 min. after, injection of the insecticides. Concentrations of acetylcholine (usually 0.125 μgm./ml.) in Ringer's solution were chosen which produced 60–80% of maximal contraction of the frog rectus preparation, and with a strict five-minute cycle the rectus was found to give reproducible contractions with these concentrations. Acetylcholine was added to control whole-blood samples to give the same concentrations, and after 60 sec. at room temperature (25° C. ± 1°) this mixture was added to the frog rectus preparation. No contractions were observed; thus all of the acetylcholine had been hydrolyzed by the blood within the incubation period. With 10 times these concentrations enough acetylcholine was left to produce a definite contraction, and higher concentrations were found to produce larger contractions. The acetylcholine stock solution (2.5 μgm./ml.) was made at the beginning of each experiment by dissolving acetylcholine chloride (Merck) in Ringer's solution acidified with hydrochloric acid to a pH of 4. The final dilution of acetylcholine was made with Ringer's solution just before addition to the bath.

This method has the advantage, as does that of Fleisher and Pope (8), of determining the amount of acetylcholine remaining after exposure to the enzyme and was considered to be especially suitable for the present investigation.

Results

(*a*) *Analysis of the Peripheral Action of Aldrin*

As shown in Fig. 2 (*A* and *B*) after double vagotomy and removal of both adrenal glands aldrin still augmented the effects on the heart of electrical stimulation of the peripheral end of the vagus nerve. An intravenous

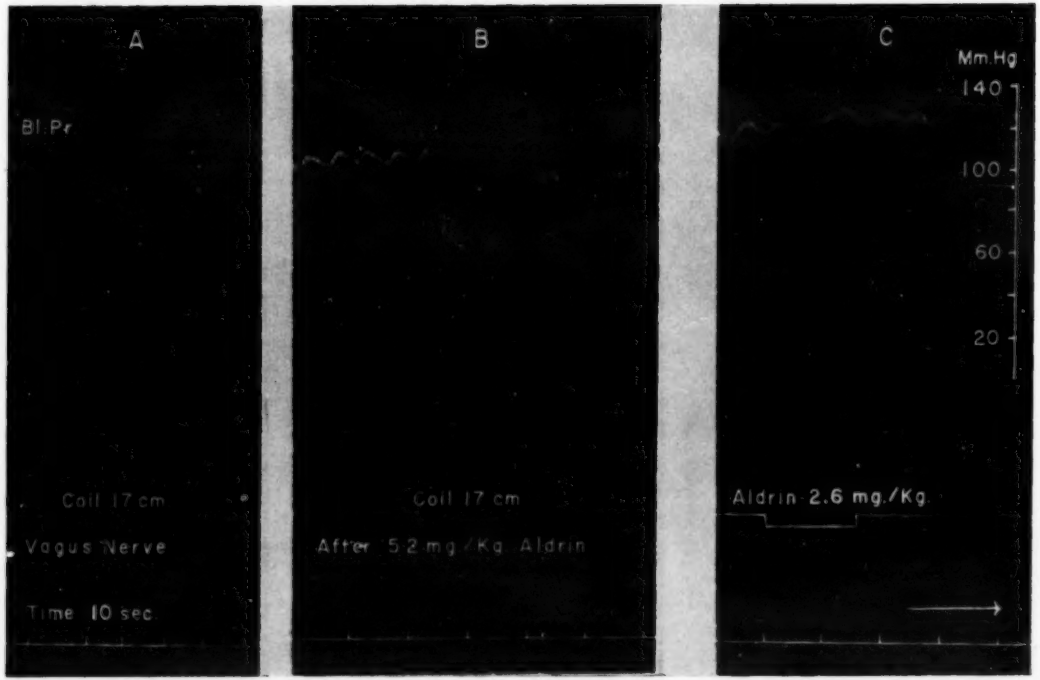

FIG. 2. Shows increased effectiveness of vagal stimulation after aldrin—cat 3.1 kgm. Double vagotomy and adrenalectomy. *A*. Stimulation of the right vagus nerve for 15 sec. before aldrin. *B*. Same stimulation repeated five minutes after the intravenous injection of aldrin. *C*. Shows transient bradycardia induced in the decentralized heart by an injection of aldrin.

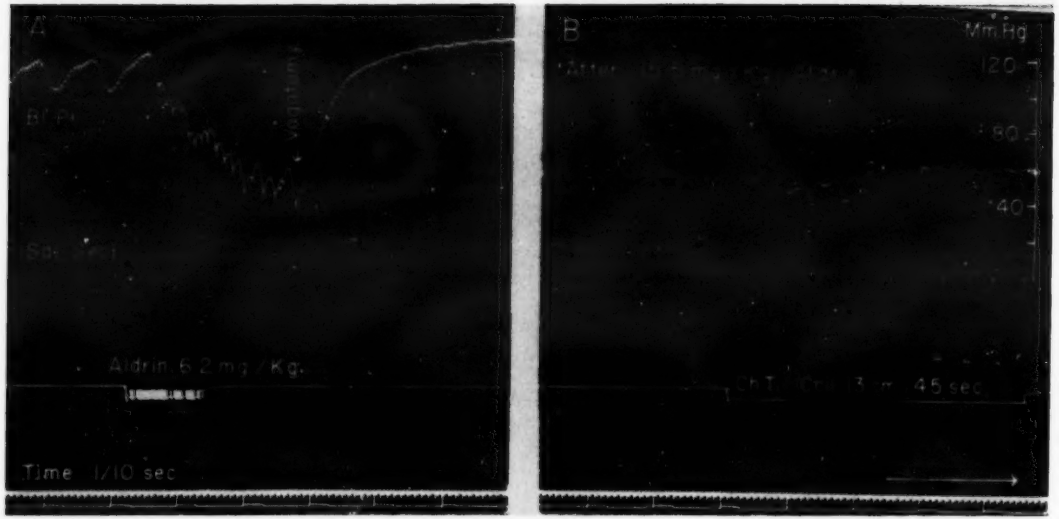

FIG. 3. *A*. Shows marked bradycardia of central origin induced by an injection of aldrin in an adrenalectomized cat (2.6 kgm.) with intact vagus nerves. *NOTE* abrupt return of the heart beat almost to the preinjection rate on section of the vagi. *B*. Shows cardiac slowing and slight lowering of the blood pressure induced by stimulation of the chorda tympani following a large quantity of aldrin (same cat, both vagi severed, and adrenals removed).

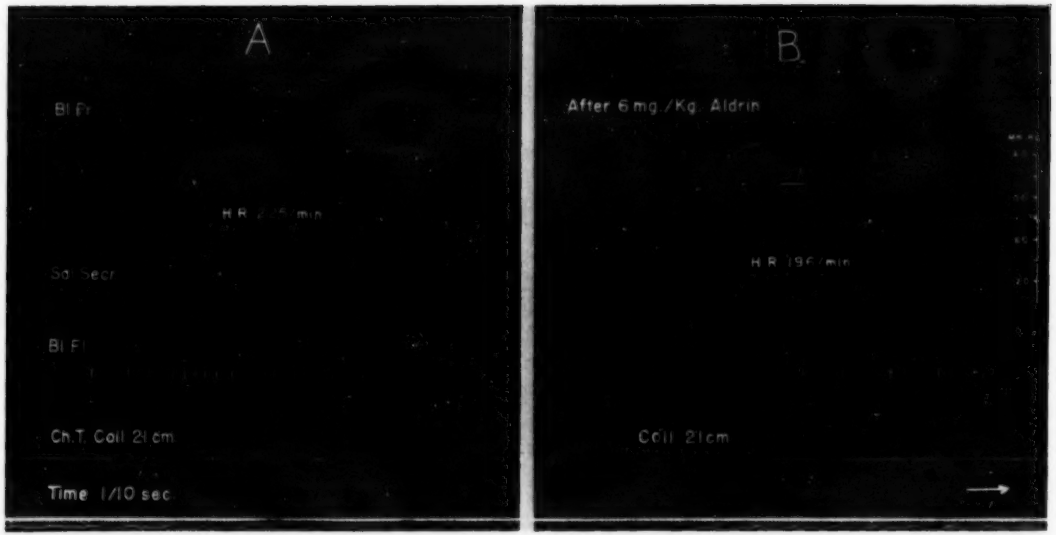

FIG. 4. (*A* and *B*). Shows increase in salivary secretion and inhibition of the heart rate on chorda tympani stimulation nine minutes after the injection of aldrin into vagotomized and adrenalectomized cat (3.3 kgm.). *NOTE* the missed heart beats in *B* following chorda tympani stimulation.

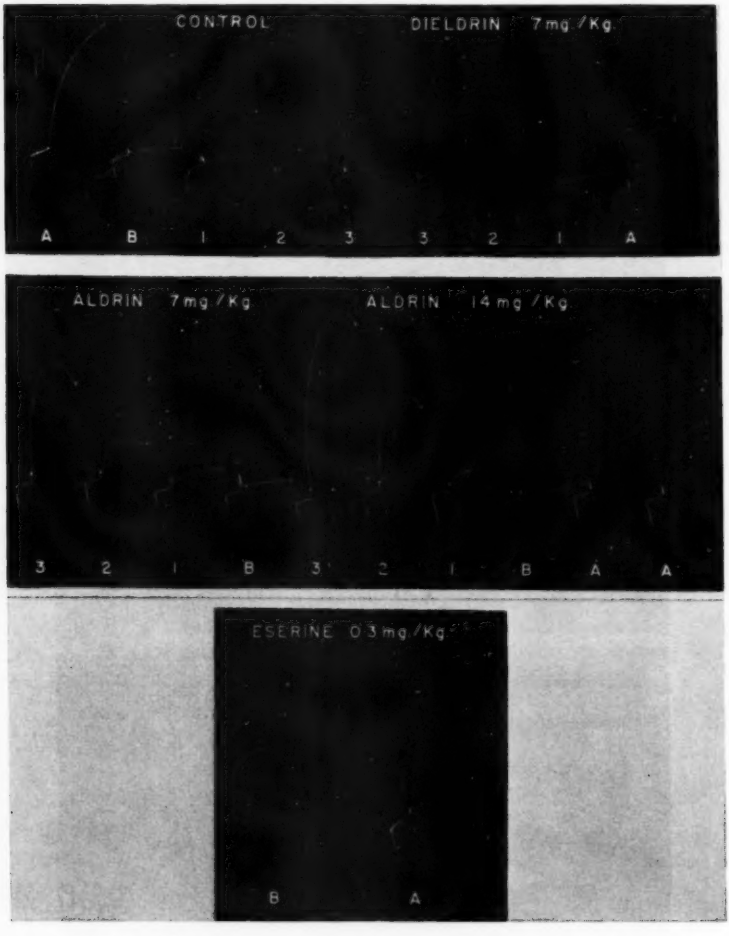

FIG. 5. Shows the rate of destruction of acetylcholine (ACh) in cat's blood as determined by the responses of the frog rectus abdominis muscle.

Abbreviations: *A*. Effect of 0.125 μgm./ml. of ACh in Ringer's solution. *B*. Effect of substitution of cat's whole blood for the Ringer's solution. 1. Effect of blood to which ACh, to a final concentration of 1.25 μgm./ml., was added 60 sec. previously. 2. Same with a final ACh concentration of 1.50 μgm./ml. 3. Same with a final ACh concentration of 1.66 μgm./ml. *NOTE* 1. Rapid destruction of ACh by the control samples of cat's blood. 2. Reduced effectiveness of ACh both in blood and in Ringer's solution after dieldrin. 3. Marked increase of responses produced by the same concentrations of ACh in the blood after intravenous injection of aldrin into the cat, and a persistence of the depression of the response to ACh in Ringer's solution. 4. Eserinized blood, ineffective by itself (*B*); completely lost power to destroy ACh, and after four applications restored the full effect of ACh solutions on the rectus (*A*).

All samples of blood were removed from the cat five minutes after each injection of dieldrin, aldrin, and physostigmine (eserine 0.3 mgm./kgm.) and tested immediately.

injection of aldrin also caused, by itself, some slowing of the heart (Fig. 2*C*); this effect was, however, much less than that observed when the vagus nerves were intact (Fig. 3*A*).

Similarly, electrical stimulation of the chorda tympani after aldrin resulted in a marked increase in the volume of secretion of saliva (Fig. 4). This was found in experiments in which, besides complete decentralization of the submaxillary gland, a bilateral vagotomy and adrenalectomy had been performed. Occasionally after large quantities of aldrin, stimulation of the chorda tympani resulted not only in a greater secretion of saliva, but also caused slowing of the heart and development of a transient cardiac irregularity similar to a vagotonic missed beat (Fig. 4*B*). The augmentation by aldrin of the vasodilator effect of stimulation of the chorda tympani reported previously (10) was also observed in the present study.

Dieldrin, in spite of a strong central action described elsewhere (11), exerted none of these peripheral effects.

(*b*) *Effects of Aldrin and Dieldrin on the Responses of the Frog Rectus Preparation to Acetylcholine*

The rate of destruction of acetylcholine by the blood of a cat before and after the intravenous administration of dieldrin and of aldrin is demonstrated in Fig. 5. When the cat's blood was introduced into the bath by itself, it did not produce any contractions of the rectus either before or after the injections of dieldrin or aldrin. Moreover, the contractions in response to acetylcholine in Ringer's solution were depressed by previous administration of blood containing dieldrin or aldrin, although in other experiments in which dieldrin and aldrin were applied directly to the rectus bath in suspension, or dissolved in corn oil, no effect was observed on subsequent contractions induced by acetylcholine in Ringer's solution. In spite of this depressant effect, the response of the rectus to the addition of acetylcholine in cat's blood containing aldrin was much greater than in the control blood. Dieldrin, however, although it depressed the muscle and its response to acetylcholine in Ringer's solution, apparently did not affect the rate of destruction of acetylcholine by the blood samples. On the other hand, when blood containing physostigmine was added to the muscle bath, the rectus completely regained its sensitivity to acetylcholine in spite of repeated, preceding applications of blood containing dieldrin or aldrin.

These results were reproduced in every experiment in which the blood was tested in this manner, and held true both in intact cats and in those which had been adrenalectomized and had the vagus nerves severed before the injection of the insecticides.

Discussion

The above experiments suggest that the aldrin-induced bradycardia and lowering of the blood pressure is the result of two different actions, whereas the effect of dieldrin involves only one. The first and most powerful effect,

common to both dieldrin and aldrin, is mediated through the vagus nerves and can be annulled by cervical vagal section. These compounds are presumably stimulating the vagal centers in the same way as they produce hyperreflexia and convulsions. Aside from this effect, however, aldrin under the conditions of the present experiments appears to have a peripheral parasympathomimetic action which, though definite, is considerably weaker than that of physostigmine. Aldrin itself produces bradycardia when it is injected after both vagi have been severed; it potentiates the effects of a given electrical stimulation of the vagus nerve or of a given dose of acetylcholine, and produces an effect with a previously subthreshold electrical stimulation or dose of acetylcholine. The responses of the completely decentralized submaxillary salivary gland (both to electrical stimulation and to injected acetylcholine) are similarly potentiated by aldrin: this is shown by considerable increases in salivary secretion. Some transmission of the effect of electrical stimulation of the chorda tympani to the circulatory system, as shown by cardiac slowing and irregularity, can also be demonstrated in these experiments. This finding is in keeping with the results described by Babkin *et al.* (3, 2) after physostigmine administration. The lowering of blood pressure which they observed was often absent in the present experiments, presumably because the blood pressure was already low owing to the large doses of aldrin and the adrenalectomy. Atropine blocks the parasympathomimetic effects of aldrin but not the convulsions or effects on striated muscle.

Potentiation by small doses of adrenaline of the effects of acetylcholine on spinal centers, the neuromuscular junction, the sympathetic ganglion, and on digestive glands has been shown by various investigators (1, 4, 5, 12, 14). Evidence of adrenal discharge after aldrin administration was obtained by Waud (15), who found that the blood glucose level in cats more than doubled after 7.5 mgm. per kgm. aldrin; when convulsions occurred, the level became even higher. Adrenalectomy prevented this rise. In the present work, the possibility that the potentiating effect of aldrin on the salivary gland and on the heart is caused not by a direct action but through the release of adrenal medullary substances has been ruled out by adrenalectomy.

In analyzing this peripheral action of aldrin the results obtained with the frog rectus preparation suggest a possible mechanism. The sensitivity and reliability of this biological method of estimating acetylcholine were carefully tested before any blood samples were added to the bath. The rectus gave consistent, submaximal contractions with concentrations of acetylcholine in Ringer's solution of the order of 0.125 μgm./ml. Samples of cat's blood, without added acetylcholine, removed both before and after injection of the insecticides produced no contraction of the rectus. This fact suggests that aldrin and dieldrin did not liberate large amounts of acetylcholine into the general circulation—a mechanism which has been considered to account for some of the effects of DDT.

The rectus muscle was found to be depressed by repeated administration of blood containing either aldrin or dieldrin: the standard contractions produced

by acetylcholine in Ringer's solution were reduced in amplitude after the muscle had been exposed to blood containing either insecticide. These contractions gradually returned to control levels after repeated washing of the muscle with Ringer's solution over some 30 min. In spite of this depression the heights of the contractions of the rectus produced by acetylcholine incubated with control whole-blood samples for 1.0 min. were greatly increased when the blood was withdrawn five minutes after the intravenous injection of aldrin. They were not increased, however, after dieldrin administration. From these observations it may be deduced that aldrin either potentiated the effect of acetylcholine on the muscle, or that it retarded the destruction of acetylcholine by the blood. As mentioned above, the contractions induced by acetylcholine in Ringer's solution were not potentiated either by previous administration of blood containing aldrin or by previous addition to the bath of aldrin suspended in Ringer's solution or dissolved in corn oil. Thus the most probable explanation seems to be that aldrin retarded the rate of destruction of acetylcholine by the blood, or in other words, it acted like an anticholinesterase. Whether this effect of aldrin is on serum cholinesterase or on that of the red corpuscles is not known.

These results are of particular interest in view of the findings of Crevier *et al.* (6). They employed the Gomori method (9) with phenyl benzoate as substrate, and reported an initial decrease of serum esterase activity in rats after poisoning with aldrin. This decrease lasted up to 10 hr., and was as pronounced as the ultimate rise in esterase activity when the aldrin was given orally in single or multiple doses. The method they employed was sensitive to common esterase, lipase, and pseudocholinesterase, and enzyme identification studies showed that the rise in esterase activity was due mainly to an increase in aliesterase. They did not, however, identify the enzyme which was concerned in the early, transient decrease of activity.

The question remains why this reduction of acetylcholine destruction was not detected by the investigators who used chemical methods. The methods are not as sensitive as the biological preparation used in this study where differences of less than 0.125 μgm./ml. of acetylcholine produced large changes in the amplitude of muscle contraction. On the other hand most chemical methods involve the addition of large excesses of the acetylcholine substrate and this excess may influence the reaction in a different way from that when the quantity of added acetylcholine is delicately balanced against the activity of the esterase. Whatever the reason may be for the discrepancy, these experiments indicate that in the early stages of acute poisoning aldrin reduces the rate of destruction of acetylcholine by cat's whole blood, and suggest that its peripheral parasympathomimetic action may be an anticholinesterase-like effect.

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DISTRIBUTION OF ADRENALINE AND NORADRENALINE IN THE NORMAL AND HYPERTHYROID RAT FOLLOWING ADRENALINE ADMINISTRATION[1]

By Jean Leduc[2], Robert Dubreuil, and Antoine D'Iorio

Abstract

Feeding of iodinated casein to rats produces marked variations in the adrenaline and noradrenaline content of heart, spleen, liver, adrenals, and blood. An impairment in the destruction of the injected adrenaline is noticed in similarly treated animals. The significance of these results with reference to the thyrotoxic process is discussed.

Introduction

There has been in recent years a number of observations suggesting that the activity of the thyroid gland is related to the metabolism of the catechol amines of the adrenal medulla, adrenaline, and noradrenaline. Spinks and Burn (17) and Trendelenburg (19) have shown that a fall of the amine oxidase activity of the liver occurs in hyperthyroid animals; conversely, thyroidectomy is followed by a rise in the activity of this enzyme (17). These studies and many others (2, 3, 10, 14, 16, 18) suggest that the augmented response to adrenaline injection in the hyperthyroid animal is to be interpreted as a fall in the rate of destruction of adrenaline in this organism. Imazumi and Kawamoto (8) found that the adrenaline dehydrogenase system of the blood was inhibited *in vitro* by thyroxine, adding weight to this interpretation. On the other hand, some studies on the catechol amine content in the adrenals of the hyperthyroid animal would seem to point to a stimulating influence of the thyroid hormone on the synthesis and/or secretion of the catechol amines by the adrenal gland (4, 6, 7, 11, 13). In order to decide between these two hypotheses we have studied the distribution of adrenaline and noradrenaline in the normal and hyperthyroid rat, using the newly described fluorometric method of Weil-Malherbe and Bone (20, 21).

Experimental

Young male rats of the Sprague–Dawley strain weighing about 75 gm. were used. The basal ration consisted of a mixture of corn meal, soybean meal, salts, corn oil, cystine, and vitamins (4). The animals were kept on this ration for four weeks. To induce hyperthyroidism the experimental group was fed a normal diet supplemented with 0.15% iodinated casein, as described by Betheil and Lardy (1). This treatment was started after one week on normal diet and lasted three weeks.

After this feeding period, the control and hyperthyroid animals were divided into six groups of at least seven animals each. One group of normal

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[2] *Summer studentship from American Cyanamid Company.*

and one group of hyperthyroid rats were anesthetized by intraperitoneal injection of Nembutal (4 mgm./100 gm.). After five minutes, the animals were bled through the abdominal aorta; the adrenals, heart, spleen, and liver were then excised. The other four groups of rats were anesthetized and each rat received, after five minutes, an intramuscular injection of *l*-adrenaline (175 μgm. in 0.5 ml.). One group of normal and one group of hyperthyroid rats were sacrificed five minutes after this injection, while the last group of normal and hyperthyroid animals were killed 15 min. after the adrenaline injection. Of the 15 hyperthyroid rats that were injected with adrenaline, two animals died in less than five minutes and three others before the end of the 15-min. period.

The pooled blood samples from each experimental group were treated according to the method of Weil-Malherbe and Bone (20, 21). The pooled organ samples were homogenized in 0.1 *N* hydrochloric acid. Immediately before the fluorometric estimations, each sample was adjusted to pH 6.5 with sodium carbonate (0.5 *N*) and 1 ml. of 1% sodium thiosulphate was added as antioxidant. After centrifugation and adjustment to pH 8.4 with acetate buffer, the samples were extracted on alumina and the eluates treated according to the method of Weil-Malherbe and Bone (20, 21) as described for plasma.

The fluorometric estimations of adrenaline and noradrenaline were made with the Farrand photoelectric fluorometer, as described by Persky and Roston (12). The adrenaline and noradrenaline values obtained with this method were reproducible for each of the organs studied. In our hands, however, the technique did not lead to an adequate differential estimation of adrenaline and noradrenaline in rat plasma; there appeared to be some interfering substance in this material. On account of this difficulty, the total fluorogenic material of the plasma was estimated as adrenaline.

Results

In the present experiment body weight was used as a criterion of hyperthyroidism. After a three-week treatment with iodinated casein the animals weighed on the average 27% less than normal rats.

The data of Table I show the effect of the treatment on the adrenaline and noradrenaline content of the adrenals, liver, spleen, and heart. In the hyperthyroid group, the adrenaline concentration per mgm. tissue nitrogen was increased in the heart (47%), spleen (18%), and liver (12.5%). In the adrenals, the concentration appeared to be decreased if calculated on a tissue nitrogen-content or weight basis, as was previously observed (4, 6, 7, 12). The adrenaline content per unit gland was, however, about the same in all the experimental groups. The noradrenaline content of the heart was considerably lower in the hyperthyroid group (−36%) but that of the spleen was increased (53%). The noradrenaline content of the adrenals, like the adrenaline content, was decreased when calculated on the basis of tissue nitrogen, but was approximately constant when calculated per unit gland.

TABLE I

EFFECT OF INJECTED ADRENALINE ON ADRENALINE (*A*) AND NORADRENALINE (*N*) CONTENT OF ADRENALS, HEART, SPLEEN, AND LIVER OF NORMAL AND HYPERTHYROID RATS

Group	No. of animals	Time after injection of adrenaline	Heart mγ/mgm.N		Spleen mγ/mgm.N		Liver mγ/mgm.N		Adrenals			
									γ/mgm.N		γ/gland	
			A	*N*	*A*	*N*	*A*	*N*	*A*	*N*	*A*	*N*
	7	No injection	3.03	15.7	2.09	9.34	0.64	1.39	16.6	3.84	10.0	2.5
Normal	9	5 min.	6.84	16.2	2.63	12.2	0.68	1.56	17.7	3.92	10.4	2.3
	9	15 min.	11.8	15.9	3.36	17.6	0.79	1.15	16.0	3.39	10.3	2.2
	6	No injection	4.47	10.0	2.46	14.3	0.72	1.41	9.8	2.1	10.9	2.3
Hyperthyroid	6	5 min.	6.14	8.87	3.05	22.1	4.46	1.94	9.3	2.3	10.5	2.6
	4	15 min.	11.0	8.81	4.27	23.5	1.45	1.19	13.9	3.1	13.5	3.0

The data that show the influence of iodinated casein on the total fluorogenic material in plasma (measured as adrenaline) are presented in Table II. The apparent adrenaline content of the plasma is reduced by about 20% in the hyperthyroid animals.

TABLE II

EFFECT OF INJECTED ADRENALINE ON THE CONTENT OF ADRENALINE-LIKE FLUOROGENIC SUBSTANCES OF PLASMA OF NORMAL AND HYPERTHYROID RATS

Group	No. of animals	Time after injection of adrenaline	Total fluorogenic material, γ adrenaline/100 ml. plasma
Normal	7	No injection	1.18
	9	5 min.	1.65
	9	15 min.	2.52
Hyperthyroid	6	No injection	0.94
	6	5 min.	3.77
	4	15 min.	4.46

The data in Tables I and II show also the effect of an intramuscular injection of adrenaline on the catechol amine content of the plasma and organs after 5 and 15 min. The myocardial accumulation of injected adrenaline was previously observed by Raab (14, 15). This effect was about the same in the two groups of animals.

In the liver of the control animals, there appeared to be a small increase in the concentration of adrenaline after the administration of this hormone; in the liver of the hyperthyroid rats, this increase was extremely marked after five minutes (560%); after 15 min., the adrenaline concentration was still about twice the initial value.

The injection of adrenaline was followed by a substantial increase in the concentration of this hormone in the spleen of the normal and hyperthyroid animals (61% and 74% after 15 min.). Surprisingly, there was also a comparable increase in the noradrenaline content of the spleen (88% and 64%). The adrenals on the other hand did not respond to this treatment and showed no important changes in their catechol amine content.

In the plasma a substantial increase in the amount of accumulated fluorogenic material followed the adrenaline injection. In the control group there was a rise of 40% after five minutes and of 114% after 15 min.; in the hyperthyroid group a threefold increase had occurred within five minutes, and after 15 min., the increase had reached 374% of the initial value.

Discussion

Goodall (6) noted that the suprarenals of thyroxine-treated sheep showed a lower adrenaline content than normal. Similar results were observed in the rat (4, 7) and in the guinea pig (13). On the other hand, Pekkarinen *et al.* (11) noted an increase in the adrenaline content of the adrenals of thyroid-fed rats: this discrepant result had not been accounted for.

In the present experiments a marked lowering of both adrenaline and noradrenaline was noted. However, if the results were expressed as catechol amine per gland rather than per unit weight there was no significant difference between hyperthyroid and normal animals. This result implies that the apparent fall in catechol amines is due only to the hypertrophy of the adrenal cortex. Eartly and Leblond (5) have shown this hypertrophy to be mediated through the hypophysis.

The fact that little or no variation of catechol amine content of adrenals, plasma, spleen, and liver occurs during hyperthyroidism rules out the hypothesis of a thyroid-stimulating effect on the adrenal medulla.

Heart muscle is the only tissue that shows a marked increase of adrenaline concentration after treatment with iodinated casein. However, the injection of adrenaline into both normal and treated rats reveals in hyperthyroid animals a more pronounced accumulation of adrenaline in all organs except the adrenals. This would suggest a reduced capacity of these tissues to catabolize adrenaline. The small reduction of amine oxidase activity in the liver of thyroxine-treated animals, reported by Spinks and Burn (17) and Trendelenburg (19), lends added weight to this hypothesis.

Feeding of iodinated casein also appears to interfere in a complex way with the metabolism of noradrenaline, the concentration of which shows a substantial decrease in the heart, an increase in the spleen, with no significant changes in the liver (9). A remarkable fact is the simultaneous increase of adrenaline and decrease of noradrenaline in the heart. This may indicate an interconversion of the two amines in this particular tissue.

An interesting observation is the large increase of noradrenaline of the spleen following an injection of adrenaline. This fact might suggest that the spleen is capable of demethylating adrenaline. However, it may also be that the Weil-Malherbe (21) method is not entirely specific and that some other fluorogenic catechol accumulates in the splenic tissue. Further work is in progress to settle these points.

Acknowledgments

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BIOCHEMISTRY OF THE USTILAGINALES

X. THE BIOSYNTHESIS OF USTILAGIC ACID[1]

By B. Boothroyd[2], J. A. Thorn[3], and R. H. Haskins[4]

Abstract

The β-cellobiolipid, ustilagic acid, deposited when *Ustilago zeae* (PRL 119) is grown in a medium containing glucose, was prepared from 1-C^{14}-glucose. The labelled acid was degraded to carbohydrate and fatty acid fractions. The carbohydrate was shown to be synthesized from 3-C fragments and the fatty acid from 2-C fragments. These results show that the hexose metabolism of the organism is probably the same as that suggested in the Embden–Meyerhof scheme.

Introduction

Previous papers in this series have been concerned with the growth of *Ustilago zeae* upon glucose media (6, 15, 20), the structure and properties of ustilagic acid, a crystalline β-cellobiolipid produced by the organism (10, 11, 12, 13, 14), and with the characterization of fermentation products from other strains of *Ustilago zeae* (7).

The fact that the organism produced a substance from which carbohydrate and fatty acid fractions could be obtained easily, together with evidence (21) that it would grow on xylose producing an identical substance, suggested its use in a series of tracer studies on fungous metabolism.

Experimental

The preparation, extraction, and preliminary degradation of the ustilagic acid followed, with slight modifications, the methods of earlier papers. The methyl-α-D-glucoside degradation was carried out by a method recently described elsewhere (2), and the degradation of the fatty acid by the method of Mendel and Coops (16). Mendel and Coops' method was used only for converting a fatty acid to its next lower homologue, but it has been adapted in this work to make possible the collection of the 1-C fragment for radioactivity measurements.

The organism used in the fermentations was a strain of *Ustilago zeae* PRL 119.

The 1-C^{14}-glucose was prepared by the method of Isbell *et al.* (8).

Radioactivity Measurements

The measurements taken on the fermentation products, and during the acid degradation, were made using plated samples either of the substance itself or of barium carbonate, in a windowless counter working in the proportional region.

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[4] *Mycologist.*

Measurements made during the glucoside degradation were made with a gas counter, similar to the one described by Buchanan and Nakao (3), standardized with the U.S. National Bureau of Standards C^{14} standard, a solution rated at 1280 d.p.s./ml.

Culture Technique

The fermentation was conducted in 50 ml. of the usual basal medium (20) containing 2.5 gm. of labelled glucose with total activity of around 15 μc. as the only source of carbon. The shake-flask and methods used were as described by Shu (18). The fermentation, judged to be complete when oxygen uptake ceased, normally took five days.

After completion of the fermentation, the flask was placed in the refrigerator overnight to ensure a more complete precipitation of the ustilagic acid. Then, before the culture mixture reached room temperature again, it was acidified with 5 ml. of sirupy phosphoric acid and shaken for 15 min. to drive off any residual carbon dioxide, which was absorbed in sodium hydroxide solution.

The sodium hydroxide solution was removed from the flask and analyzed for absorbed carbon dioxide, and for radioactivity by precipitation of barium carbonate from an aliquot. The 55 ml. of acidified culture mixture was centrifuged to bring down the cells and the ustilagic acid. The acid was then extracted from the solid cake with hot methanol (5 × 20 ml.). This methanol solution was concentrated in a current of air until cloudy, then heated to 50° C., mixed with four times its own volume of water (also at 50° C.), and allowed to cool very slowly. By this method it was possible to obtain an easily filterable precipitate of ustilagic acid. Aliquots of the centrifuged culture broth and of the cells were combusted by the method of Thorn and Shu (22) for radioactivity measurements.

Degradation of Ustilagic Acid

The ustilagic acid (297.1 mgm.), which had been filtered onto paper for counting, was degraded by treatment with sodium hydroxide (0.1 *N*; 10 ml.) for one hour. The glucoustilic acid, which resulted from this degradation, was further broken down with methanolic hydrogen chloride (3%, 25 ml.) to give methyl-α-D-glucoside and a mixture of methyl ustilates A and B (10, 11).

The methyl-α-D-glucoside sirup (89.9 mgm.) from the degradation did not crystallize on cooling. Therefore it was diluted with inactive substance (581.1 mgm.) and the whole recrystallized from methanol after it was decolorized with animal charcoal.

The methyl esters (92 mgm.) (Fig. 1) obtained on evaporation of chloroform extract from the methanolysis were dissolved in dry acetone (5 ml.) and a few drops of concentrated sulphuric acid. The solution was allowed to stand for five hours at room temperature and was then poured into water. The two isopropylidine derivatives (I, II) were extracted with chloroform. After

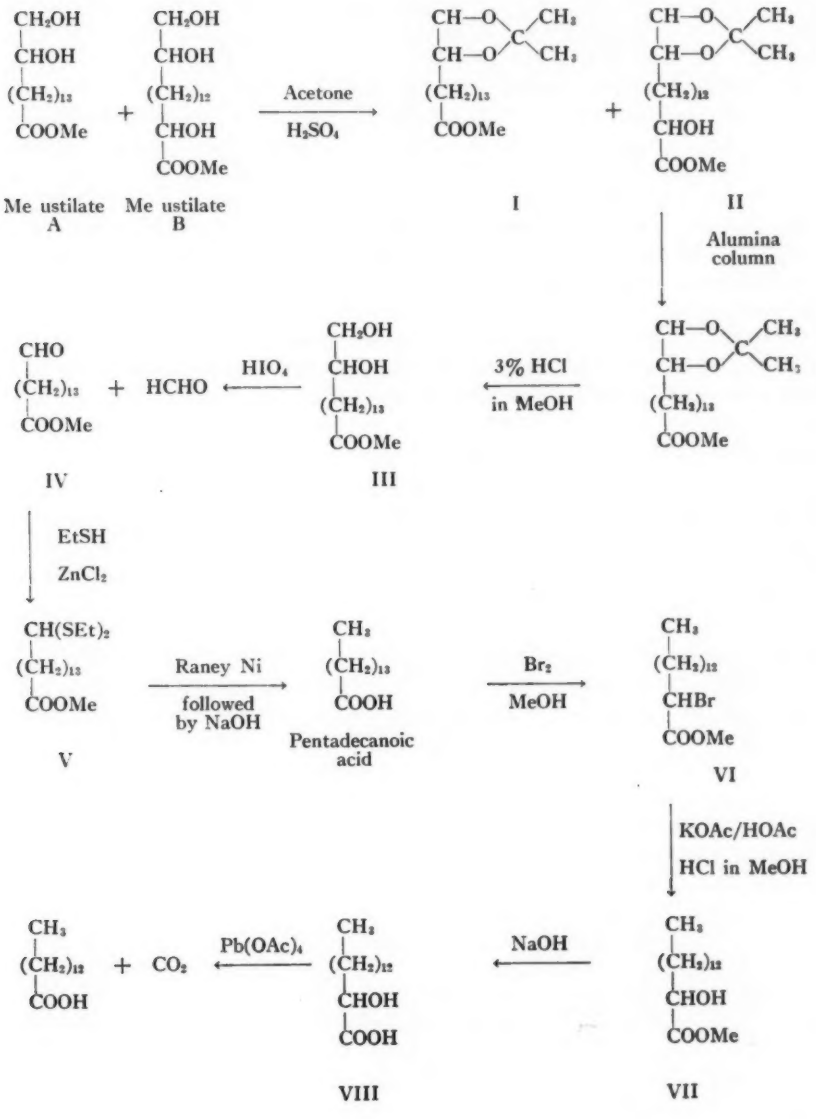

FIG. 1. Isolation and degradation of methyl ustilate A.

the extract was washed with water and dried over anhydrous sodium sulphate, the chloroform was removed by evaporation leaving a colorless solid (97.3 mgm.). To separate the two derivatives, this solid was dissolved in benzene (10 ml.), run onto an alumina column (15 × 1.6 cm.) prepared by packing as a benzene slurry, and washed through with a further volume of benzene (65 ml.). Isopropylidene methyl ustilate B (II) with a free hydroxyl group did not pass through the column, whilst the isopropylidene methyl ustilate A (I) washed through easily. Evaporation of the benzene left a colorless solid which was refluxed for two hours with methanolic hydrogen chloride (3%; 10 ml.). After the resultant solution was poured into water, the methyl ustilate A was extracted with chloroform (3 × 15 ml.). When the chloroform solution was washed and dried and then freed from chloroform by evaporation, there remained an almost pure sample of methyl ustilate A (41.7 mgm., m.p. 84.5°–85° C.). A pure sample (11) melts at 85.5°–86° C. The over-all yield of methyl-α-D-glucoside from the ustilagic acid was 81%, and of methyl ustilate A, assuming this to be 70% of the ustilic acid mixture, 52%.

Preparation of Pentadecanoic Acid

The purified active methyl ustilate A was diluted with seven times its own weight of inactive substance and recrystallized from 75% aqueous methanol giving colorless plates (317.2 mgm.) m.p. 85°–86° C. A measured portion was suspended in water and plated on a filter paper disk for a determination of radioactivity.

Purified methyl ustilate A (205 mgm.) was dissolved in methyl alcohol (10 ml.) and periodic acid (0.4 *M*; 3 ml.). The mixture was allowed to stand for one hour, diluted with water (50 ml.), and the oil which deposited extracted with benzene (3 × 30 ml.). The aqueous layer containing the formaldehyde from C_{16} of the ester (III) was treated with excess calcium carbonate, and filtered to remove the calcium periodate and calcium iodate which precipitated. The cleared solution was now mixed with a 1% solution of dimedon in phosphate buffer (2) (10 ml.). A precipitate of the formal/dimedon derivative separated (183 mgm. 92.5%) and was filtered off and recrystallized from 50% aqueous ethanol before it was plated for counting.

The benzene extract containing the methyl aldehydopentadecanoate (IV) was dried with anhydrous sodium sulphate and the solvent blown off in a stream of nitrogen at room temperature. The residue was allowed to stand for 12 hr. with ethyl mercaptan (3 ml.) and freshly fused zinc chloride (0.5 gm.). The excess mercaptan was removed in a stream of air, and the mercaptal (V) purified by solution in ether and thorough extraction with water. The sirup which remained after removal of the ether was placed in a vacuum oven at 60° C. for five hours to remove the last traces of mercaptan.

The mercaptal (V) was refluxed for six hours with 75% aqueous ethanol (50 ml.) and freshly prepared Raney nickel catalyst (1) (5 gm.). The solution after filtration through diatomaceous earth, was evaporated down and the residue saponified with sodium hydroxide solution. The acid, which was

recovered by acidification and ether extraction, was recrystallized once from 50% aqueous ethanol, m.p. 51°–53° C. Pentadecanoic acid 53°–54° C., mixed m.p. with authentic sample, 52°–53° C. Yield 100.4 mgm. (61%).

In an earlier test, better yields of both the formaldehyde (100%), and the pentadecanoic acid 96% were obtained.

Degradation of Pentadecanoic Acid to Tetradecanoic Acid and Carbon Dioxide

This method, which follows the work of Mendel and Coops (16), gave in optimum cases an over-all yield from one acid to the next lower one of 75%, when used at a 200–300 mgm. scale.

Active pentadecanoic acid (100.4 mgm.) was mixed with inactive acid (200.8 mgm.) and the whole treated with red phosphorus (0.05 gm.) and bromine (0.28 gm.) as described by Mendel and Coops. The resultant sirup was mixed with methanol (4 ml.) and a pale yellow oil methyl α-bromo-pentadecanate [442.2 mgm. 100%] (VI) obtained. Treatment of this, first with a mixture of potassium acetate and acetic acid, and then with methanolic hydrogen chloride (3%) gave the α-hydroxy ester, which, instead of being purified by distillation, was immediately converted to the acid. Any unconverted pentadecanoic acid was removed by washing with petroleum ether, which left a colorless solid (231.4 mgm.).

The apparatus, used for degradation of the α-hydroxy acids with lead tetraacetate, consisted of a closed three-necked flask with a stirrer and oxygen inlet in one neck, a dropping funnel in another, and a condenser leading to a bead tower on the third. The oxygen was passed first through a sodium hydroxide solution to remove any carbon dioxide, then through concentrated sulphuric acid solution to remove water. The inlet was arranged so that it was below the surface of the liquid in the flask, to ensure good mixing. The condenser was used on the take-off arm, because the reaction which takes place in benzene was conducted at 50° C. The bead tower contained carbonate-free sodium hydroxide (0.2 *N*; 25 ml.) to trap the carbon dioxide formed during the reaction.

The α-hydroxy-pentadecanoic acid (VIII) (231.4 mgm.) was dissolved in dry benzene (20 ml.) and dry ether (10 ml.) and then added slowly to a solution of lead tetraacetate (0.84 gm.) in dry benzene (20 ml.). The addition was complete in one hour and the reaction was then allowed to run for a further hour before a few drops of glycerol were added to decompose the excess lead tetraacetate. Before the solution cooled, it was filtered to remove the lead diacetate and lead dioxide, which was then washed with warm benzene. Glacial acetic acid (2 ml.) was added to the mixed filtrate and washings and the whole evaporated to dryness to remove any paraformaldehyde formed from the glycerol. The residue was taken up in 30°–60° C. petrol ether and washed carefully with water. It was then extracted from the petrol ether solution with two 25-ml. portions of alcoholic potassium hydroxide (0.5 *N* in 50% aqueous alcohol). Acidification of this solution precipitated a colorless solid which was extracted with ether. The extract was dried by anhydrous

sodium sulphate, and evaporated to give tetradecanoic acid (189 mgm.) as an oil which crystallized on standing. The over-all yield was 67%.

The carbon dioxide formed during the reaction was precipitated as barium carbonate and plated for radioactivity determination. The procedure was repeated three times to remove carbons 1, 2, and 3 from the pentadecanoic acid.

Results and Discussion

Two and one-half (2.5) grams of 1-C^{14}-glucose in 50 ml. of basal medium were fermented by *Ustilago zeae* in five days.

Total radioactivity was 13.3 μc.

Analysis of broth revealed 0.85 gm. of glucose had not been fermented. (See Table I.)

TABLE I

RECOVERY OF RADIOACTIVITY

Portion of fermentation	Radioactivity, μc.	Carbon, gm.	μc./gm.
CO_2	4.81	0.327	14.71
Broth*	5.90	0.337	17.5
Cells	0.94	0.109	8.62
Ustilagic acid	1.53	0.167	9.16
Total	13.18	0.940	

μc./gm. of C_1 in 1-C^{14}-glucose = 87.5.
Gm. of carbon in 2.5 gm. glucose = 0.927.
** A large part of the activity (4.5 μc.) can be accounted for by unused 1-C^{14}-glucose.*

The carbon dioxide formed during the fermentation had a specific activity approximately one-sixth of that in C-1 of the glucose and would therefore appear to have been formed equally from all six carbon positions. (See Table II.)

TABLE II

DISTRIBUTION OF C^{14} IN GLUCOSE OF USTILAGIC ACID

Carbon atom in glucoside	Activity in diluted sample, μc./mM.	%
1	0.055	75.1
2	0.00115	1.57
3	0.00157	2.14
4	0.000554	0.76
5	0.00052	0.71
6	0.0122	16.7
Total	—	96.98

Note: Total activity in diluted glucoside 0.0732 μc./mM.

The data in Table II show that, although a large part of the glucose from the medium was incorporated into the ustilagic molecule without scission of the carbon chain, some of the glucose was obtained by resynthesis from smaller molecules. The results can be explained by the initial reactions of the Embden–Meyerhof scheme (9). If it is assumed that glucose was converted to the equilibrium mixture of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate then any glucose resynthesized from these trioses by a reversal of the reactions would be labeled equally in C-1 and C-6. The amount of glucose resynthesized by this mechanism would then be $2 \times 16.7\% = 33.4\%$ while the amount incorporated without scission would be $75.1 - 16.7 = 58.4\%$. These two portions then account for 91.8% of the ustilagic acid glucose.

The degradation of methyl ustilate A (see Table III) showed that the carbon atoms in even-numbered positions had a much higher activity than the odd-numbered carbon atoms. This agrees with the work of Popjak (17) on the biosynthesis of milk fat in the rabbit and also with the investigations of Dauben *et al.* (5) on fat biosynthesis in the mouse. The results are what would be expected if methyl-labeled pyruvate, formed from glucose-1-C^{14} by the reactions of the Embden–Meyerhof scheme, gave rise to "active acetate" which then condensed to build up the fatty acid chain, as demonstrated with animals (19). It was not practical to make a complete degradation of the methyl ustilate but it appears that the activity is uniformly distributed through the carbon chain, for if the average activity of C-1 and C-3 is taken to be that of the odd-numbered carbon atoms and the average activity of C-2 and C-16 that of the even-numbered carbon atoms, summation gives a figure of 107.2% for the whole molecule.

TABLE III

DISTRIBUTION OF C^{14} IN METHYL USTILATE

Carbon no.	Activity, μc./mM.	% of total
C_{16}	0.0988	12.5
C_1	0.00846	1.07
C_2	0.0922	11.6
C_3	0.0129	1.63

These investigations show that the biosynthesis of fats and carbohydrates by *U. zeae* probably does not differ radically from that of other organisms. The authors do not know of any similar investigation on other fungi although Coleman *et al.* (4) have shown that acetate is incorporated in fatty acids by *Fusarium lini.*

Acknowledgments

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THE METABOLISM OF *ASCARIS LUMBRICOIDES* OVARIES

I. NITROGEN DISTRIBUTION[1]

By John K. Pollak[2] and Donald Fairbairn

Abstract

The protein fraction obtained from *Ascaris lumbricoides* ovaries was analyzed for total, α-amino, and amide nitrogen, as well as amino acids and nucleic acids. In the corresponding non-protein fraction total, α-amino, amide, and ammonia nitrogen, and free amino acids and urea, were determined. Free and protein amino acids were qualitatively similar, but quantitatively dissimilar. Unusually large amounts of proline and alanine were found in the proteins, whereas arginine and methionine could not be identified in protein or free acids. Glutamic acid and alanine comprised one-half of the free acids. Ammonia was present in relatively high concentration, but urea was absent. Much of the non-protein nitrogen was not identified. Ribo- and desoxyribonucleic acids were found in approximately the relative proportions occurring in rat liver, but in much lower concentration. The existence of phosphoproteins was not clearly established. Glycogen and total reducing substances, however, were present initially in high concentrations which decreased markedly when the parasites were maintained in a non-nutrient medium.

Introduction

Nitrogen metabolism in nematode parasites has never been investigated in detail, nor has there been any adequate description of the nitrogenous constituents of the various organs and tissues. The total nitrogen content of several species was reported by Weinland (48), Flury (22), Smorodintzev and Bebeshin (43), Clavera and Mallol (9), and Cavier and Savel (8), and the amino acid composition (in part) by Flury (22) and Yoshimura (50). More recently, Rogers (38) identified polypeptides, ammonia, and urea as nitrogenous excretion products in three species of intestinal nematodes.

For the investigation of nitrogenous substances and their metabolism, *Ascaris lumbricoides*, a parasite inhabiting the pig's intestine, appeared to be suitable since it is relatively large and readily available. The ovaries of this species were selected for study because egg production is large (11) and probably continuous throughout the female parasite's mature life; hence they could be assumed to be metabolically active with respect to nitrogen. Before undertaking an investigation of ovarian metabolism, however, it was desirable to ascertain the nature and amounts of the nitrogenous constituents. The present communication is concerned only with this aspect of the problem.

Methods

Living specimens of ascaris were collected at the slaughter house and maintained in the laboratory in a non-nutrient medium (3) for a period not

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exceeding 24 hr. The ovaries of mature females were obtained by slitting the body wall longitudinally, carefully removing the intestine, and separating the oviducts and uteri. They were then blotted free of perienteric fluid and weighed. The paired ovaries of 395 individuals varied in weight between 130 and 716 mgm. (averaging 363 mgm.) with solids accounting for 31% of the wet weight. As a rule about 2 gm. of pooled tissue were taken for analysis.

Quantitative analyses were made for (1) total nitrogen, (2) protein nitrogen, (3) non-protein nitrogen (NPN), (4) α-amino and amide nitrogen of protein and NPN, (5) ammonia nitrogen in NPN, (6) amino acids of protein and NPN, (7) nucleic acids, and (8) glycogen and total reducing substances. Since the accuracy of many of these analyses depended upon the effective precipitation and separation of proteins from NPN, analyses in groups 1–5 inclusive were performed on ovary homogenates treated with three different protein precipitants. A detailed description of these procedures follows.

Approximately 2 gm. of ovaries were homogenized in 5 ml. of ice-cold trichloroacetic acid (5%), ethanol (75%), or tungstic acid (five parts of 10% sodium tungstate mixed with seven parts of 0.6 *N* sulphuric acid) (42). The glass tube and pestle of the homogenizer were fitted to tolerances producing cell-free preparations after one to two minutes homogenization. The homogenates were then diluted with the appropriate reagent to give 10% suspensions, mixed well, and portions taken for total nitrogen determinations. The remainder of the homogenate was centrifuged for 10 min. at 1800 *g* and the supernatant decanted and diluted to the desired volume. Portions of this solution were taken for analysis of total NPN, α-amino nitrogen, amide nitrogen, and ammonia. Very often the NPN solution was slightly opalescent rather than clear. Experiment showed, however, that the insoluble matter producing the opalescence could be removed at the centripetal pole by high-speed centrifugation (20,000 *g*) and that it was nitrogen-free. The protein sediment remaining after removal of the NPN was suspended without additional washing in 20 volumes of 6 *N* hydrochloric acid, and hydrolyzed by refluxing for 24 hr. at 120° C. The hydrolyzate was then analyzed for total nitrogen, ammonia (amide) nitrogen, and α-amino nitrogen.

Total ovarian nitrogen, protein nitrogen, and NPN were determined on samples containing 20–100 μgm. nitrogen, by acid digestion according to Ma and Zuazaga (34) followed by nesslerization with the Koch and McMeekin reagent (29). Ammonia nitrogen in the NPN fraction, and in neutralized protein hydrolyzate (amide nitrogen), was determined by the Conway micro-diffusion method (10). α-Amino nitrogen of the NPN fraction and neutralized protein hydrolyzate was converted to ammonia by means of the ninhydrin reaction (44) and the ammonia determined by microdiffusion. Amide nitrogen in the amino acids of the NPN fraction was hydrolyzed according to Speck (45) and determined as ammonia. Urea was estimated manometrically by Krebs' method (32), and phosphorus colorimetrically (19).

A more elaborate procedure was employed in preparing ovaries for the analysis of amino acids. Ethanolic homogenates were diluted as previously

described, and set aside at 4° C. overnight. After the suspension was centrifuged at 1800 g for 10 min. the protein sediment was washed twice with 3 ml. of 75% ethanol, and the combined extracts were washed with three volumes of chloroform in order to remove undesirable substances (1). The solution was then dried *in vacuo* over sulphuric acid, and the residue dissolved in sufficient water to make a solution containing approximately 1.5 mgm. NPN per ml. Meanwhile, the protein sediment was hydrolyzed as previously described, and the hydrolyzate filtered free of humin and dried *in vacuo* over sodium hydroxide pellets. The residue was then dissolved in water to make a solution containing about 5 mgm. of nitrogen per ml.

Hydrolyzed samples of NPN were prepared when required by mixing 0.1–0.2 ml. portions of the NPN solution with an equal volume of 6 *N* hydrochloric acid and heating at 120° C. for 24 hr. in a sealed capillary tube. The cooled hydrolyzate was placed on a watch glass and diluted with two 0.2 ml. washings from the tube. This solution was then dried over sodium hydroxide as above and the residue redissolved in a volume of water equal to the volume of NPN solution originally hydrolyzed.

Amino acids in NPN and hydrolyzed protein fractions were determined by ascending chromatography (15) on Whatman No. 1 paper at 26 ± 1° C. In the first dimension the solvent used was butanol – acetic acid – water (4 : 1 : 1, v/v) after equilibration of the chromatography chamber and paper for 6–12 hr. with solvent and a beaker of water. The second dimension was developed with phenol–water (3 : 1, w/v) after equilibration with water alone, the solvent being placed in the troughs just before development was started. The usual color developer was a 0.25% solution of ninhydrin containing 0.02% stannous chloride, but a number of other reagents were also used as occasion demanded. Quantitative determinations were performed by Fowden's method (23) and employing Fowden's modification of the Moore and Stein ninhydrin reagent. Absorbancy of the colored solutions was determined at 570 mμ except for proline, which was determined at 440 mμ. Comparison of unknowns and standards was made by reference to standard curves constructed for 18 amino acids, 16 of which gave a linear response for amounts not exceeding 0.4 μgm. α-amino nitrogen per ml. of final solution. Of the remaining two acids, tyrosine and cysteine, tyrosine was estimated by making use of Fowden's data (23) for the molecular extinction coefficient compared with that of leucine. Blank determinations were made on each chromatogram.

Those amino acids which were readily separated by one-dimensional chromatography were determined with reasonable accuracy (93–107% recoveries) by the above method. Some acids, however, were not always clearly resolved by one-dimensional development, and after development in the second dimension with phenol–water, losses (10–40%) were encountered during drying of the paper, in confirmation of the findings obtained by Brush *et al.* (6). Accordingly, the results obtained from quantitative determinations of two-dimensional chromatograms were corrected in conformity with the

recovery of the corresponding standard solution used in the same experiment. Such results, which were less accurate than those obtained from one-dimensional chromatograms, are clearly indicated in the tabulated data.

Nucleic acids were determined by the Schmidt and Thannhauser procedure (39). Glycogen, total reducing substances, and fermentable reducing substances were determined according to the method of Glocklin and Fairbairn (24).

Results

The results obtained when protein (including nucleic acids and phospholipids) and NPN fractions were separated by three different protein precipitants are presented in Table I. Clearly, ethanol, trichloroacetic acid, and tungstic acid were about equally efficient in precipitating proteins, since the values for total nitrogen, α-amino nitrogen, and amide nitrogen in this fraction were essentially independent of the nature of the precipitant employed. Marked differences were observed, however, between ethanol and trichloroacetic acid on the one hand, and tungstic acid on the other, in the characteristics of the NPN. Thus, NPN obtained by tungstic acid fractionation contained greatly reduced amounts of total NPN and of all NPN components which were determined. Tungstic acid is said to precipitate water-soluble peptones and polypeptides (28), but these substances were not demonstrable in chromatograms of the NPN obtained by ethanolic precipitation of proteins. Since a large fraction of the total NPN obtained by all three methods was not accounted for by α-amino acids, urea, or ammonia, it seems possible that much

TABLE I

DISTRIBUTION OF NITROGEN IN *Ascaris lumbricoides* OVARIES

Fraction*	Protein precipitant		
	Ethanol (75%)	Trichloroacetic acid (5%)	Tungstic acid (10%)
	Mgm. N per 100 gm. ovaries		
Total N	1700	1581	1583
Protein N	1508	1373	1425
α-Amino N	981	861	959
Amide N	116	121	119
Non-protein N	85	113	27
α-Amino N	20.1	23.5	9.9
Amide N	1.4	1.1	0.5
Ammonia N	2.7	2.9	1.5

* *Mean values (duplicate analyses) obtained from two batches of ovaries, in which variation in the different fractions and subfractions did not exceed ± 6% of the respective means, except in the case of total N, in which the maximum variation was ± 8%, and of the non-protein N and its subfractions in the tungstic acid precipitation, in which the maximum variation was ± 16%.*

of the unidentified NPN was precipitated by tungstic acid, its appearance in the protein fraction being effectively masked by the large excess of protein present.

Paper chromatography revealed the presence of 16 and 14 identifiable amino acids in the non-protein and protein fractions, respectively. In the non-protein fractions obtained from two ovary homogenates (Table II), proline, phenylalanine, threonine, tyrosine, and cysteine were each present in amounts representing less than 0.5% of the total amino acid nitrogen. Amino acids of the protein hydrolyzates were qualitatively similar to non-protein acids, except for the absence of glutamine and asparagine, which were probably present in the native protein, since the hydrolyzate contained considerable amounts of ammonia (amide) nitrogen (Table I). A faintly positive Hopkin's-Cole test for tryptophane, which is also destroyed by acid hydrolysis, was obtained in unhydrolyzed aqueous homogenates. The non-protein chromatograms showed one minor unidentified ninhydrin-positive spot, and the protein

TABLE II

OCCURRENCE AND DISTRIBUTION OF AMINO ACIDS IN *Ascaris lumbricoides* OVARIES

Amino acid*	Free amino acids		Protein amino acids	
	Mgm. α-amino N/100 gm. ovaries	% Total α-amino nitrogen	Mgm. α-amino N/100 gm. ovaries	% Total α-amino nitrogen
Leucine	0.3	1.5	190	12.7
Glutamic acid	6.5†	33	163†	10.8
Serine + glycine	2.9†	15	156†	10.4
Proline	Trace	<0.5	122	8.2
Aspartic acid	0.5†	2.5	121†	8.0
Lysine	2.0	10	120†	8.0
Valine	0.3	1.5	117	7.8
Alanine	3.3	17	107	7.1
Histidine	1.6	8.2	87†	5.8
Phenylalanine	Trace	<0.5	51	3.4
Threonine	Trace	<0.5	46†	3.1
Tyrosine	Trace‡	<0.5	62‡	4.1
Cysteine	Trace	<0.5	Present	
Asparagine	0.5	2.5	—**	—
Glutamine	1.0	5.1	—**	—
Arginine	None		None	
Methionine	None		None	
Tryptophane	Trace**		Trace**	
Total	18.9		1342	
Total α-amino nitrogen in fraction	19.7		1505	
Recovered as α-amino N (%)		96.3		89.4

* *Mean values obtained from two experiments in which the variation did not exceed ± 7% of the respective means. Qualitative identification was made in a total of six experiments.*

† *Two-dimension chromatograms.*

‡ *Determined indirectly (see Methods).*

** *See the text.*

chromatograms two. These spots still appeared when the developed and dried chromatograms were dusted with basic copper carbonate before being sprayed with ninhydrin (12) and so were presumed to be α-amino acids.

Histidine, with an R_f value similar to arginine, was identified by the Pauly test (4) which was used also to confirm the presence of tyrosine. Arginine could not be detected on the paper by the specific phenol–hypochlorite reaction (4) nor could methionine by the sensitive permanganate oxidation described by Dalgliesh (13). The latter test was useful also in the identification of several other amino acids. A preliminary acid hydrolysis of the non-protein fraction resulted in the disappearance of glutamine and asparagine from the chromatograms, and corresponding increases in the amounts of glutamic and aspartic acids. No other changes were observed, from which it appeared likely that the non-protein fraction did not contain significant amounts of soluble peptones and polypeptides.

Quantitatively, marked differences were observed between the amino acids present in the non-protein and protein fractions. In the former, glutamic acid and alanine accounted for 50% of the total acids, and serine, glycine, lysine, and histidine for another 33%. Protein amino acids were more evenly distributed, with leucine being most abundant. Glycine and serine were only partially separated on the chromatogram, and were determined together. No error was introduced in this way, since the two acids have identical extinction coefficients in the Moore and Stein color reaction. In the protein fraction, however, the determined amounts of these acids is undoubtedly somewhat high, owing to the presence of small amounts of serine known to occur in the phospholipids (37) and to glycine formed by purine breakdown during acid hydrolysis (35). In the non-protein fraction the combined values for glutamine and asparagine were slightly higher than the values which can be calculated from the amounts of acid amide nitrogen present in the ethanol extract of a different batch of worms (Table I). This is not surprising, for under the conditions employed for amide hydrolysis, glutamine is completely hydrolyzed, whereas the hydrolysis of asparagine is incomplete (31).

There appeared to be no important qualitative difference in the nature of the amino acids obtained from distal and proximal halves of the ovaries; or from the uteri, which are filled with fertilized but unembryonated eggs. Although quantitative studies were not made, these chromatograms were similar to those already described.

Nucleic acid and phosphoprotein phosphorus was determined in four different batches of ovaries (Table III) and compared with results obtained by analysis of rat liver. The latter agreed well with those obtained in other laboratories (39, 41). Ovarian ribo- and desoxyribonucleic acids were found in proportions similar to those of the liver acids, but in smaller (40%) concentration. Phosphoproteins appeared to be virtually absent. Unlike liver desoxyribonucleic acids, the corresponding ovary acids were only partially extracted (25–40%) by hot trichloroacetic acid (40) from the alkali-soluble, acid-insoluble fraction obtained by the Schmidt-Thannhauser method. The

TABLE III

NUCLEIC ACID AND PHOSPHOPROTEIN PHOSPHORUS IN *Ascaris lumbricoides* OVARIES

(Milligrams P per 100 gm. tissue by method of Schmidt and Thannhauser (39))

	Total acid-insoluble P	Phosphoprotein P	Ribonucleic acid P	Desoxyribonucleic acid P‡
Ovaries*	52 (48–59)	1.3 (0.0–2.5)	41 (36–46)	9.8 (4.8–13.5)
Rat liver†	131	0.0	106	25

** Average of four experiments. Extreme values are given in parentheses.*
† Single experiment.
‡ By difference. In one experiment, direct determination of total phosphorus in this fraction confirmed the value obtained by difference.

significance of this difference in response to acid is not understood. Total phosphorus (52 mgm.) in the Schmidt-Thannhauser alkaline digest was equivalent to 87 mgm. of nucleic acid nitrogen, if calculated from the data of Fletcher *et al.* (20) for ribonucleic acid. Thus, nucleic acids represented a significant fraction of the unrecovered protein nitrogen apparent from examination of Table I.

The fact that the nitrogenous components of ascaris ovaries accounted for only 35% of the tissue solids (see Discussion) is explained in part by the high glycogen concentration in this organ (Table IV). It is well-known that surviving ascarides utilize a part of their glycogen stores (5), and this utilization apparently includes ovarian stores, since ovaries of freshly-collected parasites contained nearly twice as much glycogen as those obtained from worms subsequently maintained for three days in a mineral medium. From Table IV it is clear that glycogen comprised most of the total reducing substances, all of which were fermented by washed yeast.

TABLE IV

CHANGES IN GLYCOGEN AND TOTAL REDUCING SUBSTANCES OF OVARIES (IN GM. PER 100 GM. OVARIES) DURING SURVIVAL OF *Ascaris lumbricoides* IN A NON-NUTRIENT MEDIUM

Time (days)	Glycogen	Total reducing substances*
Fresh	7.1	7.7
1	5.4	7.0
3	4.2	5.3

** Determined with the Nelson copper reagent after acid hydrolysis of the tissue (24). Completely fermented by yeast.*

Discussion

If the total protein nitrogen of ascaris ovaries (Table I) is expressed in terms of protein by using the conventional factor (6.3), it is found to comprise only 35% of the total tissue solids. The remaining solids consist mainly of glycogen and other reducing substances (25%, Table IV) and lipids (30–35%) (17). This calculated protein content is only approximate, however, since the figure for total nitrogen includes 15–20% of non-protein, phospholipid, and nucleic acid nitrogen (Tables I and IV, and data of Rogers and Lazarus (37) for phospholipids). Even so, the concentration of nitrogen in the ovaries exceeds that in ascaris muscle, which was found by experiment and conversion to terms of protein to comprise 7.1% of the fresh muscle weight, or 28% of the muscle solids.

α-Amino nitrogen accounted for 65% of the ovarian protein nitrogen, and amide nitrogen for 8%. The remainder included nucleic acid and phospholipid nitrogen, as well as nitrogen contributed by diamino, aromatic, and heterocyclic amino acids. It is probable that most, if not all, of the amide nitrogen was present originally in glutamine and asparagine (14).

Several points concerning the protein amino acids may be mentioned. The absence of arginine and methionine, and the relatively large concentrations of proline and alanine, are features unusual in animal tissues (16, 49). Failure to detect arginine was indeed so unusual that evidence more conclusive than that provided by paper chromatography alone should be sought (see Addendum). The results are in general agreement with the lim ted data obtained for the whole worm by Flury (22) and Yoshimura (50), who had less sensitive methods at their disposal. The abundance of leucine and valine is interesting because of their possible metabolic relationship with the volatile hexanoic and pentanoic acids, which are the major products of ascaris fermentation (7), and which are found in considerable quantities in the ovary triglycerides (17). However, it must be emphasized that the analytical methods used were unsuitable for identification of the individual leucine isomers, and that the specific nature of the hexanoic acids remains unknown.

Qualitatively, the free amino acids of ovaries were similar to those reported to be present in the perienteric fluid by Kajihara and Hashimoto (27) after the present investigation was completed. The free amino acid concentration in ovaries is similar to that found in vertebrate tissues (2, 25, 26), although the amide nitrogen (glutamine and asparagine) is much lower (25). Comparison with invertebrate tissues is not possible, for with the exception of the hemolymph of several species, data are unavailable. In ascaris, there are striking quantitative differences between free and protein amino acids. Leucine, valine, and proline, for example, made up less than three per cent of the free acids, but more than 30% of the protein acids. The situation is reversed with respect to glutamic acid and alanine, which account for 50% of the free acids, and for less than 20% of the protein acids. The importance of glutamic acid and alanine in the protein metabolism of the ovaries is the subject of another communication (36).

Urea has been established as a minor end product of nitrogen metabolism in three intestinal nematodes by Rogers (38) who states also that ascaris ovaries contain little urease (and arginase). This observation is in accord with our failure to find urea (and arginine) in the non-protein fraction. Rogers found that ammonia was a major excretion product in the same species, and it was also shown in this laboratory (unpublished experiments) to be the main waste product of nitrogen metabolism in ascaris. The concentration of ammonia nitrogen occurring in ovaries (nearly 3 mgm.%) is very high when compared with vertebrate tissues, and is also higher than the concentrations found in the hemolymph of certain snails and lobsters (21) and of the horse botfly (33). Analysis of the hemolymph (perienteric fluid) of ascaris revealed the presence of ammonia in a concentration (2.5 mgm.%) similar to that of the ovaries. Although it is probable that most of this ammonia represents waste nitrogen, evidence has been gathered to show that it may also function as a substrate in the amination of pyruvate to alanine and aspartic acid (36).

The results obtained in the analyses for nucleic acids (Table IV), while apparently clear-cut, cannot be considered to be accurate until confirmed by other analytical methods. The apparent ratio of cytoplasmic to nuclear acids (4 : 1) is in accord with the preponderance of ribonucleic acids usually found in protein-synthesizing tissues or organs, but is much lower than the ratio occurring in unfertilized sea-urchin eggs (30).

Glycogen in *Ascaris lumbricoides* ovaries was comparable to the amounts found by Toryu in *A. megalocephala* ovaries (47). Undoubtedly this glycogen, like the lipids (18), serves as an energy store to be drawn on during subsequent embryonation of the eggs. It is probable, however, that some if it is used together with ovarian nitrogen, in the formation of the N-acetylglucosamine residues of chitin, which is laid down in the egg shell following fertilization (46).

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Addendum

One of us (J.K.P.) has now found that the ovaries of *A. lumbricoides* collected at Sydney contain arginine in both the protein and free amino acid fractions. The small amounts present, representing about three per cent of the total nitrogen in each fraction, were detected by the sensitive method of Eden *et al.* (Australian J. Exptl. Biol. Med. Sci. 32 : 333. 1954.) It may be mentioned, however, that since the distribution of nitrogen in the Australian ascaris was quantitatively different from that obtained in the present investigation, it would be unwise to conclude without additional evidence that arginine was present also in the Canadian ascaris.

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THE METABOLISM OF *ASCARIS LUMBRICOIDES* OVARIES

II. AMINO ACID METABOLISM[1]

By John K. Pollak[2] and Donald Fairbairn

Abstract

Homogenates of ascaris ovaries contained transamination, deamination, and reductive amination systems. Alanine–glutamic and aspartic–glutamic transaminases were active, whereas weaker transaminations occurred between glycine or serine and α-ketoglutaric or pyruvic acids. Sixteen other amino acids did not participate in transamination with these keto acids. A minor reaction, however, occurred between alanine or glutamic acid and α-ketovaleric acid. No amino acid oxidase activity was detected, and deamination appeared to be limited to a weakly positive glutamic dehydrogenase, which could be coupled to the alanine–glutamic and aspartic–glutamic transaminases to form a transdeaminase system. In the presence of pyruvate and ammonium chloride reductive amination occurred and alanine and aspartic acid were synthesized. This reaction was accelerated by bicarbonate, although oxalacetate could not be substituted successfully for pyruvate in the amination system. The results of the investigation are in accord with the probability that an active protein synthesis occurs in ascaris ovaries.

Introduction

The nitrogenous components in the ovaries of *Ascaris lumbricoides* were investigated recently (15), with results which show that glutamic acid and alanine together comprised some 50% of the total free amino acids, and that the free ammonia concentration was unusually high. It was of interest, therefore, to examine ascaris ovaries for the presence of enzymatic systems involved in transamination, deamination, and reductive amination. Such studies might be expected to contribute to the understanding of the protein metabolism occurring in the ovaries of this prolific egg-producing parasitic nematode. There appears to be no record of any similar investigation in ascaris or in other species belonging to the phylum Nematoda.

Methods

The ovaries were isolated as described previously (15) from female *A. lumbricoides* collected at the slaughter house less than 24 hr. previously. Between 1 and 2 gm. of tissue (four to six ovaries) were homogenized in 5 ml. of iced *M*/15 Sørensen phosphate buffer (pH 7.4 or 7.8) with the Potter-Elvehjem apparatus, the pestle of which was selected to provide essentially cell-free preparations in one minute. Routine microscopic examinations were made in order to check the uniformity of the homogenates used in different experiments. When ovaries were to be used for the study of deamination (glutamic dehydrogenase) the phosphate buffer was fortified with *M*/10

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nicotinamide before the tissue was homogenized. Portions of each homogenate were taken for the determination of total nitrogen by kjeldahl digestion and subsequent nesslerization (15). In the study of amino acid oxidases, both homogenates and minces were used. The latter, prepared from the threadlike ovaries cut into approximately 0.5 cm. lengths in a cooled mortar and then suspended in buffer, were considered to simulate the tissue slices ordinarily used in the examination of other tissues for these enzymes.

All substrates were obtained commercially, except for sodium pyruvate, which was prepared according to Robertson (17). They were prepared in $M/10$ solutions and neutralized just before use where necessary. The amino acids were chromatographically pure except cysteine and glutamine, the latter containing traces of glutamic acid and asparagine. When racemic amino acids were used they were prepared as $M/10$ solutions with respect to the *l*-isomer.

In the study of transamination and reductive amination Awapara and Seale's method (2) for the preparation and incubation of reaction mixtures and for subsequent protein precipitation was employed. The resulting ethanolic extracts of non-protein nitrogen were evaporated to dryness and the residue dissolved in 1 ml. of water. Portions of this solution were then chromatographed quantitatively on filter paper as previously described (15), with both the disappearing and the newly synthesized amino acids being determined. Standard amounts of the relevant amino acids were chromatographed simultaneously, in order to check the efficiency of the method in each experiment.

Oxygen uptake and carbon dioxide production were measured manometrically by Warburg's direct method. Dehydrogenases (including glutamic dehydrogenase) were determined manometrically by ferricyanide reduction according to Quastel and Wheatley (16). In this method the acidity resulting from the reduction of ferricyanide by the dehydrogenase systems is measured by carbon dioxide evolution in the presence of excess bicarbonate. All experiments were carried out at 37° C.

Results

Transamination

The reliability of the chromatographic method for the determination of amino acids and therefore of transaminases was established when it was shown that 50 μmole each of glutamic acid, aspartic acid, or alanine, when added to homogenates without addition of α-keto acids, could be recovered quantitatively (95–105%) after a one-hour incubation period. Endogenous free α-amino nitrogen, amounting to only 0.75 μmole per 0.5 ml. of homogenate, was not a serious source of error, nor was the presence of small amounts of glutamic dehydrogenase (Table VI).

Both alanine and aspartic acid were actively transaminated in the presence of α-ketoglutarate. The reverse reactions, i.e., transamination of glutamic acid in the presence of pyruvate and oxalacetate, also occurred (Table I).

TABLE I

TRANSAMINATION IN OVARY HOMOGENATES OF *A. lumbricoides**

Amino acid substrate	α-Keto acid substrate		
	Ketoglutarate	Pyruvate	Oxalacetate
	$Q_{T(N)}$ (μM. amino acid transaminated/mgm.N/hr.)		
Glutamic	—	8.1	18.5
Alanine	15.3	—	2.1
Aspartic	10.6	1.3	—
Glycine	0.8	0.4	—
Serine	0.4	0.4	—

* *Each tube contained 0.5 ml. homogenate, 0.5 ml. M/10 amino acid, 0.5 ml. M/10 α-keto acid, and 1.5 ml. M/15 phosphate buffer (pH 7.4). Incubated one hour at 37° C.*

A much slower reaction occurred between alanine and oxalacetate, or pyruvate and aspartic acid. It has been shown in mammalian tissues (13, 14) that these slow reactions may arise from the combined actions of the more powerful alanine–glutamic and aspartic–glutamic transaminases. Among the other amino acids tested (arginine, asparagine, cysteine, cystine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine, and valine), only glycine and serine served as amino-group donors in the presence of ketoglutarate or pyruvate, and even in these cases the reactions were sluggish. It was also observed that a third acid, α-ketovaleric, was transaminated by glutamic acid and alanine, but the amounts of (presumptive) norvaline formed were not sufficiently large to be determined quantitatively. The function of other keto acids as amino group acceptors, which might have considerable physiological significance, was not investigated.

It is clear that active alanine–glutamic and aspartic–glutamic transaminases were present in ascaris ovaries, and that these reactions were reversible. The data summarized in Table I do not, however, show that each system was catalyzed by a specific enzyme. Evidence of such specificity was obtained in two ways. First, it was readily shown that each system was saturated with substrates at the concentrations employed (50μmole per 3 ml. of reaction mixture), i.e., further increase in substrate concentration did not increase the rate of reaction. This being the case, then addition of aspartate to the alanine–glutamic acid system, or of alanine to the aspartic–glutamic system, would not be expected to increase the reaction rate if a common enzyme catalyzed both reactions. If, however, specific enzymes were involved, then the observed rate should be equal to the sum of the two individual rates.

It is seen by examination of Table II, that the rates were, in fact, additive. Tables II, III, and IV also indicate that the rates were approximately constant only during the first few minutes, although under ideal conditions constancy should prevail so long as the substrate concentrations remained sufficiently high to keep the enzymes saturated.

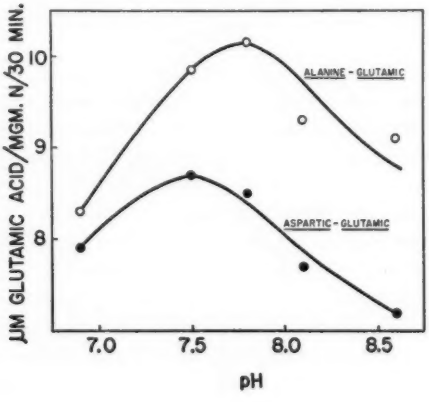
TABLE II

THE ADDITIVE EFFECT OF THE TRANSAMINATION OF ALANINE AND ASPARTIC ACID WITH α-KETOGLUTARATE*

Additions	Amount, μM.	Glutamate formed, μM., in: 15 min.	30 min.
l-Alanine	50		
α-Ketoglutarate	50	6.7	10.2
l-Aspartate	50		
α-Ketoglutarate	50	4.9	6.7
l-Alanine	50		
l-Aspartate	50	10.3	15.5
α-Ketoglutarate	100		

** Each tube contained 0.5 ml. of homogenate, amino acid, and α-ketoglutarate as indicated, and M/15 phosphate buffer (pH 7.4) to make 3 ml.*

Secondary evidence for the existence of specific enzymes catalyzing the two major transaminations was derived from a determination of the pH optimum for each reaction. It was found (Fig. 1) that the optimum for alanine–glutamic transaminase occurred at pH 7.8, and that for aspartic–glutamic transaminase at pH 7.4. For each system the forward and reverse reactions had the same optimum. In these experiments the reaction mixture was



prepared by mixing 0.5 ml. of homogenate with 0.5 ml. each of amino acid and neutralized keto acid and adding 1.5 ml. of *M*/15 phosphate buffer previously adjusted to the desired pH, which did not change significantly during the 30 min. incubation period.

In experiments involving either the forward or reverse reactions of alanine–glutamic transaminase the disappearance of added amino acid always corresponded closely with the formation of new amino acid (Table III). Judging by the initial rates (0 to 30 min.) the formation of glutamic acid from alanine and α-ketoglutarate proceeded somewhat faster than the formation of alanine in the reverse reaction. The forward and reverse reactions catalyzed by aspartic–glutamic transaminase, on the other hand, were more complex, for in these, and particularly in the reverse reaction, alanine appeared in significant amounts (Table IV). In the later stages of such experiments (60 to 480 min.) the alanine was apparently formed at the expense of aspartic

TABLE III

ALANINE–GLUTAMIC TRANSAMINASE*

	Forward reaction			Reverse reaction		
Time, min.		Alanine decrease, μ M.	Glutamic acid increase, μ M.		Glutamic acid decrease, μ M.	Alanine increases, μM.
15	Alanine and ketoglutarate	5.3	6.4	Glutamic acid and pyruvate	4.5	4.2
30	as substrates	9.3	8.4	as substrates	7.0	6.3
60		12.9	13.6		9.5	10.0
120		17.8	19.4		11.7	11.1
180		19.0	21.6		11.8	10.8

** Each tube contained 0.5 ml. homogenate, 0.5 ml. M/10 l-amino acid, 0.5 ml. M/10 α-keto acid, and 1.5 ml. M/15 phosphate buffer (pH 7.8).*

TABLE IV

ASPARTIC–GLUTAMIC TRANSAMINASE*

	Forward reaction				Reverse reaction			
Time, min.		Aspartic acid decrease, μ M.	Glutamic acid increase, μ M.	Alanine increase, μ M.		Glutamic acid decrease, μ M.	Aspartic acid increase, μ M.	Alanine increase, μ M.
15	Aspartic acid	4.2	4.7	—	Glutamic acid	6.5	7.7	1.5
30	and ketoglutarate	9.0	8.0	—	and oxalacetate	9.0	10.8	3.3
60	as substrates	14.9	12.7	—	as substrates	22.7	17.2	4.8
120		16.1	14.3	—		26.2	17.9	8.8
480		36.6	22.8	11.2		25.0	15.2	10.0

** Each tube contained 0.5 ml. homogenate, 0.5 ml. M/10 l-amino acid, 0.5 ml. M/10 α-keto acid, and 1.5 ml. M/15 phosphate buffer (pH 7.4).*

TABLE V

ALANINE–GLUTAMIC AND ASPARTIC–GLUTAMIC TRANSAMINASES IN HOMOGENATES OF ASCARIS MUSCLE AND OVARIES*

	Glutamic acid synthesized			
	μmole/gm. wet weight/hr.		μmole/mgm. N/hr.	
Transaminase	Muscle	Ovaries	Muscle	Ovaries
Alanine–glutamic	95	289	10.6	15.3
Aspartic–glutamic	129	231	14.4	10.6

** Each tube contained 0.5 ml. homogenate, 0.5 ml. M/10 l-amino acid, 0.5 ml. M/10 α-ketoglutarate, and 1.5 ml. phosphate buffer (pH 7.4). Incubated one hour at 37° C.*

acid, since the sum of the amounts of these two amino acids formed corresponded with the amount of glutamic acid which disappeared. This feature of the reaction was conveniently explained by assuming that the unstable oxalacetate lost carbon dioxide spontaneously with formation of pyruvate, which was then aminated to alanine in the presence of glutamate by the alanine–glutamic transaminase. In the terminal stages of these experiments the aspartic acid actually decreased in amount, owing possibly to the fact that α-ketoglutarate formed in the above side reaction was by that time sufficiently abundant to cause a noticeable reversion in the aspartic–glutamic transamination.

The investigation on transamination was briefly extended to include ascaris muscle, in order to provide some basis for comparison of metabolic activity in different ascaris tissues. Only the forward reactions in the two major transaminations were examined. Because the total nitrogen in muscle was found to be much less than that in ovaries, transaminase activity was expressed in terms of total nitrogen, and also of wet weight (Table V). The activity in muscle was much less than that of ovaries on a wet weight basis, but of the same order as ovarian activity if the results were expressed in terms of total nitrogen. The relative rates of the forward and reverse reactions in the two transaminations appeared to be reversed in these two tissues. In muscle, as in ovaries, no transamination occurred between pyruvate or α-ketoglutarate and the amino acids arginine, asparagine, cysteine, cystine, glutamine, histidine, lysine, methionine, phenylalanine, and proline. Pyridoxal phosphate, which is the coenzyme or coenzyme precursor for transaminases, had no effect when added in concentrations as high as 250 μgm. per ml. of reaction mixture. Presumably, therefore, the mixture was already saturated with respect to coenzyme.

Oxidative Deamination

Homogenates or minces of ascaris ovaries consumed oxygen in similar amounts, comparable to those reported previously by Laser (12). For homogenates, the mean Q_{O_2} (wet weight) was 0.17 (Q_{O_2} (nitrogen) = 10.3).

Five amino acids (*dl*-alanine, *dl*-aspartic acid, *dl*-lysine, *dl*-valine, and *dl*-phenylalanine) commonly used as substrates in the study of amino acid oxidases were without effect on the oxygen consumption of homogenates or minces. It was concluded, therefore, that neither *d*- nor *l*-amino acid oxidase was present in such preparations. On the other hand, both glutamine and glutamic acid mildly stimulated oxygen consumption, from which it appeared that glutamic dehydrogenase was present. Additional evidence was obtained by examining this reaction anaerobically, in the presence of ferricyanide and bicarbonate. In such experiments (Table VI) it was established:

(*a*) that ovaries contained an active group of endogenous dehydrogenases,

(*b*) that addition of glutamic acid increased the dehydrogenase activity significantly, and

(*c*) that the two major glutamic acid-producing transaminases in ovaries could be coupled with glutamic dehydrogenase to form a transdeaminase system.

TABLE VI

ENDOGENOUS DEHYDROGENASES, GLUTAMIC ACID DEHYDROGENASE, AND A COUPLED TRANSAMINASE–DEHYDROGENASE SYSTEM IN ASCARIS OVARIES*

Substrates	Dehydrogenase activity, μM. CO_2 evolved per hr.	
	Total	Total minus endogenous
Endogenous	4.4	—
l-Glutamic acid	5.5	1.1
l-Alanine	4.7	0.3
l-Aspartic acid	4.4	0.0
l-Alanine + ketoglutarate	5.5	1.1
l-Aspartic acid + ketoglutarate	5.3	0.9

* *Each flask contained 2.0 ml. homogenate (in M/10 nicotinamide); 0.5 ml. M/15 phosphate buffer (pH 7.8) or 0.5 ml. substrate; 0.02 M ferricyanide and 0.028 M bicarbonate (final concentration). Total volume 3 ml. Gas phase 93% nitrogen – 7% carbon dioxide.*

Reductive Amination

Reductive amination was proposed by Kritzman (11) to explain the observation that rat liver suspended in phosphate buffer synthesized aspartic acid and alanine when pyruvate, ammonium chloride, and carbon dioxide were also present. This system apparently occurred also in ascaris ovaries, as shown in the following type of experiment. Homogenates were prepared in phosphate or in phosphate–bicarbonate buffer (*M*/15 phosphate, four parts; *M*/6 sodium bicarbonate, one part) and placed in Warburg flasks which

TABLE VII

REDUCTIVE AMINATION IN ASCARIS OVARIES*

Substrates	Increment in α-amino nitrogen, μM.			Net increment, μM.
	Alanine	Glutamic acid	Aspartic acid	
Pyruvate (120 μM.)	+ 1	− 1.5	− 0.7	− 1.2
Pyruvate + HCO_3^- (100 μM.)	+ 0.4	− 1.5	0.0	− 1.1
Pyruvate + NH_4Cl (80 μM.)	+ 2.95	− 0.7	+ 1.28	+ 3.53
Pyruvate + NH_4Cl + HCO_3^-	+ 3.70	− 1.5	+ 2.27	+ 4.47

** Each flask contained 3 ml. homogenate prepared in phosphate or phosphate–bicarbonate buffer (see the text). Incubation time, two hours.*

contained alkali in the center wells if a carbon dioxide-free atmosphere was required. After addition of appropriate substrates (Table VII) the flasks were incubated for two hours, and their contents then examined chromatographically for any increment in amino acids over the control values. In such experiments it was found that in the presence of pyruvate and ammonium chloride both alanine and aspartic acid were synthesized and that this synthesis was increased in the presence of bicarbonate. In all experiments, including the controls, glutamic acid decreased, owing probably to the activity of transaminases and glutamic dehydrogenase. When pyruvate and ammonium chloride, with or without added bicarbonate, were present, the synthesis of alanine and aspartic acid greatly exceeded the decrease in glutamic acid. It was concluded, therefore, that reductive amination of pyruvate and of oxalacetate (or its metabolic equivalent synthesized by carboxylation of pyruvate) had occurred. The observed synthesis of aspartic acid in the presence of pyruvate and ammonium chloride, without added bicarbonate, may be partially explained by the fact that the phosphate buffer used was subsequently found to contain about 1 μmole of carbon dioxide per ml. It was also of interest that oxalacetate and ammonium chloride could not be substituted for pyruvate, carbon dioxide, and ammonium chloride in the synthesis of aspartic acid. This unexpected result was previously observed by Fowler and Werkman (8) in their investigation of amination by bacteria. No positive effect of adenosine triphosphate was noted in any reaction involving reductive amination by ascaris; nor is the ATP concentration in the ovaries known.

Discussion

In a large body of work concerned with mammalian and bacterial transaminases it has been shown repeatedly that in such tissues the aspartic–glutamic transaminase is much more active than the alanine–glutamic transaminase (5). The same trend was observed by Daugherty (6) in the liver

fluke, *Fasciola hepatic* (Trematoda), and by Aldrich *et al.* (1) in the tapeworm, *Hymenolepis diminuta*. Ascaris ovaries (and muscle) therefore constitute an exception to previously studied tissues, since in ascaris tissues the activity of the two transaminases is more nearly equal, and probably in favor of the alanine – glutamic acid enzyme. In this reversible system, furthermore, the rate of reverse reaction (formation of glutamic acid) exceeds that of the forward reaction, in disagreement with the equilibrium constant carefully established by Krebs (10), who used a purified enzyme preparation essentially free of side reactions. The emphasis on glutamic acid synthesis observed in ascaris ovaries is of interest in connection with the relative amounts of glutamic acid and alanine (2 : 1) occurring in the free amino acids of the ovaries (15), and with the fact that these two acids comprise one-half of the total free α-amino nitrogen. It seems reasonably certain that in ascaris ovaries, as in the tissues of other organisms, specific enzymes are involved in the transamination of alanine or aspartic acid with α-ketoglutarate. It is of interest, too, that although no transamination could be detected between valine and α-ketoglutarate or pyruvate, a similar (but reversed) reaction, in which norvaline was synthesized in the presence of α-ketovalerate, occurred to a limited extent. Reactions such as this, which would lead to the formation of a variety of amino acids, deserve further study.

Failure to identify *d*- or *l*-amino acid oxidases in ovary homogenates, or in minces (which in this case were comparable to tissue slices as ordinarily prepared) is supported by the low flavin content (2 μgm. per gm. wet weight) reported by Gourévitch (9). On the other hand, failure of added pyridoxal phosphate to stimulate the transaminases may possibly be explained by the large amounts of pyridoxin occurring in ascaris (3). The stimulation of oxygen uptake by glutamic acid and glutamine was specific for these substances, and was undoubtedly due to the presence of a glutamic dehydrogenase which was so weakly active as not to interfere seriously with the transamination experiments.

Reductive amination, on the contrary, proceeded at a rate which suggests that it might be of some importance in the nitrogen metabolism of the ovaries, particularly since it is already known (15) that these contain abnormally large amounts of ammonia. The fact that no amination occurred when oxalacetate was substituted for pyruvate must be explained for the moment by assuming that the synthetic oxalacetate used was biologically inactive, a phenomenon previously observed by Fowler and Werkman (8) in bacteria. Very recently Daugherty (7) has shown that reductive amination occurs in *Hymenolepis diminuta*, an intestinal tapeworm which was found by Chandler (4) to be able to grow even when protein was entirely omitted from the diet of its host, the rat. From the present study the conclusion may be drawn that the ovaries of *A. lumbricoides* can aminate or transaminate α-keto acids much more readily than they can oxidatively deaminate α-amino acids. The apparent emphasis on anabolic reactions is in accord with the active protein synthesis which is assumed to occur in the ovaries of this prolific parasite.

Acknowledgment

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THE DEMONSTRATION OF ACETYL PHOSPHATIDE (PLASMALOGEN) IN THE DEPOT FAT OF FOWL TREATED WITH DIETHYLSTILBESTROL[1]

By W. P. McKinley, H. Grice, and M. R. E. Connell

Abstract

The abdominal depot fat from young cockerels, male castrate fowl, or male turkeys which have been treated with diethylstilbestrol, or from hens in egg production, contains a material which responds to the Feulgen plasmalogen test. After treatment with mercuric chloride under specified conditions, fat from such birds reacts with fuchsin – sulphurous acid to give a purple colored product. The material responsible is considered to be plasmalogen. Fat from corresponding regions of non-laying hens, cockerels and male turkeys of varying ages, and castrated males does not give the reaction when it is performed under the specified conditions. A technique based on these findings is described and has been used to distinguish chickens or turkeys which have been treated with estrogen from birds which have not been thus treated.

Introduction

The effects of the administration of diethylstilbestrol and other estrogens to poultry in simulating the results of caponization have been described by a number of workers (3, 7, 11, 14), and Bird *et al.* (1) have shown the presence of residual estrogen in treated birds. The methods available for determining whether or not poultry have been treated with estrogens require a time consuming process of extracting the tissues, and subsequent injection of the extracts into ovariectomized or immature rats (4) or else chemical determination of the estrogen (8, 9).

Stepp, Feulgen, and Voit (16) demonstrated that egg yolk contained plasmalogen (an acetyl phosphatide). Feulgen and others (5, 6) later estimated that egg phosphatides contained approximately 0.1% of this material. The serum of the laying hen and estrogenized males or females contains a complex consisting of phosphoprotein (11), phospholipid, and plasmalogen (12) among other constituents. Electrophoretic patterns suggest that the serum of the laying hen, the sexually immature, estrogen treated bird, and egg yolk all contain a phospholipid–phosphoprotein complex with similar physical properties (13). The ovary of a hen in egg production probably utilizes this complex as yolk material, but there is no apparent analogous mechanism in the estrogen treated, non-laying bird for disposal of this complex.

Sinclair (15) estimated the amount of choline and non-choline containing phospholipids in the serum of turkeys and several species of animals. He obtained on one occasion a fraction which gave the Feulgen reaction for acetalphosphatides and considered this fraction to be a contaminant of the acetone insoluble fraction. He made no mention of studying this fraction further and did not state the lipid source in which this material was detected.

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The object of the present paper is to describe a rapid chemical method based on the reaction between a constituent of the abdominal fat and fuchsin – sulphurous acid which will distinguish male fowl that have been treated with estrogen from untreated birds.

Experimental

Selection and Treatment of the Birds

Chickens were obtained from a reliable source to ensure that the birds had not been treated with estrogenic material. The birds were placed in wire cages and given water and a commercial diet* ad libitum. The estrogen was administered by a subcutaneous implantation either of pellets each containing 12 mgm. of diethylstilbestrol or of a commercially prepared paste containing 10 mgm. of this estrogen. Abdominal fat was taken from the birds after slaughter in most instances, while in a few cases biopsies were made on the live birds after they were anesthetized with nembutal. Each sample was taken with clean utensils in order to avoid contaminating the fat of one bird with that of another. Only fat that was free from extraneous tissue was used.

Methods

Approximately five grams of fat was placed in a small funnel, which was fitted into the neck of a suitable flask. The flask was placed in an oven at 80° C. for a period long enough (10 to 15 min.) to render approximately 3 ml. of the fat. Excessive heating was avoided in order to prevent changes in the structure of the fat which produces materials that give a pseudo color.

One milliliter of the rendered fat was added to each of two, clean, dry, 25 ml. volumetric flasks. One milliliter of 0.05 molar mercuric chloride was added to one flask while the other served as a blank. The flasks were shaken and placed in an oven at 80° C. for exactly three minutes. They were removed, cooled, and 15 ml. of *freshly prepared* Schiff reagent was added to each. The development of a purple† color in the fat layer of a mercuric chloride treated sample indicated that the fat was from an estrogen treated bird or a laying hen. The purple color developed within seven minutes or less with fat samples containing plasmalogen. The fat from birds not treated with estrogen did not react to give a colored product. There was no color development in the samples that had not been treated with mercuric chloride unless they were allowed to react for a period longer than the time specified. The acid in the Schiff reagent will release the aldehyde by hydrolysis of the plasmalogen if given sufficient time.

In order to obtain reliable results, it was found necessary to standardize the technique of preparing the reagent, of rendering the fat, and of carrying out the test.

* *The diet consisted of mixed cereal grains and Master fattening pellets, Toronto Elevators Ltd.*

† *A deep yellow or orange color due to the carotenoids in the depot fat from a few samples masked the purple color to the extent that the resultant color appeared white.*

Schiff Reagent

The most useful reagent was prepared so as to have the same chemical composition as that described by Langley (10), but with alterations in the technique of preparation as follows:

Five grams of potassium metabisulphite ($K_2S_2O_5$) was added to 1 liter of 0.15 *N* hydrochloric acid and the mixture was shaken until the salt had dissolved. The flask was placed on a magnetic mixer and 5 gm. of basic fuchsin‡ was added slowly. The flask was stoppered immediately to prevent loss of sulphite and the stirring was continued until the dye had dissolved completely. The reagent was then placed in a refrigerator for 12 hr.

The clear, amber colored solution was placed on a magnetic mixer and 3.5 gm. of freshly activated wood charcoal† was added. The flask was stoppered and stirring was continued for one hour. The solution was filtered as rapidly as possible through a No. 1 Whatman filter paper and the flask stoppered. The reagent was clear and colorless at this stage and ready for use.

Results

The express purpose of this study was to test the depot fat from as many birds of known history as possible in order to establish the reliability of the test. The mean figures for weight gains and for liver and testes weights were calculated but these figures were of no particular interest in the present study. The response to estrogen was indicated in each bird by an increase in body and liver weight and a decrease in testes weights.

The relative intensity of the color produced by the reaction product is indicated by plus signs. The number of plus signs was assigned by visual observations of the color intensity.

The results obtained from a group of cockerels 16 weeks of age are recorded in Table I. These birds all responded to treatment, but most of the secondary sex characteristics were returning to normal at the end of the fourth week of treatment. The fat of all except one of the treated birds in this group gave a positive plasmalogen test, although the external appearance of these birds was similar to that of the control birds. The one bird which gave a very slight plasmalogen test had large testicles and the comb and wattles had returned to near normal in color and size.

The results of testing the abdominal depot fat of a group of cockerels 10 weeks of age are also recorded in Table I. The average testicle size for both the treated and untreated birds was less than those of the older birds (Table I), and there did not appear to be a very definite correlation between testicle size and plasmalogen content of the depot fat from this group of birds. A distinct test for plasmalogen was obtained in the depot fat from all treated birds of this group and plasmalogen was detected in one bird 53 days after the implantation of the pellet. There was considerable variation in the relative

† *Fisher Scientific powder, U.S.P. X.*

‡ *Basic fuchsin—certified for use in the Feulgen reaction, National Aniline Division New York.*

TABLE I

COCKERELS OF THE AGE DESIGNATED WHEN INJECTED WITH A PELLET OF DIETHYLSTILBESTROL

No. of birds	Age (wk.)	Sperm smears	Dosage (mgm.)	Days on test	Test for plasmalogen
Cross bred cockerels (Barred Plymouth Rock female × Rhode Island Red male)					
5	16		–	22–35*	All negative
1	16		12	22	++
1	16		12	30	++
1	16		12	30	+
1	16		12	35	+
1	16		12	35	Very slight
Barred Plymouth Rocks					
7	10	+	–	14–35*	All negative
2	10	–	–	21	Both negative
1	10	–	12	14	+++
3	10	–	12	21	++
1	10	–	12	35	++
3	10	–	12	35	+
1	10	–	12	53	+
White Leghorns					
6	8	+	–	21–35*	All negative
2	8	–	12	21	+++
1	8	–	12	35	+++
1	8	–	12	35	++

* *Control birds were slaughtered on the same days as the treated birds.*

intensity of the purple colored product in the fat, which would seem to indicate a variation in the plasmalogen content of the fat. The variation appeared to depend on the length of time the birds had been on treatment. The Barred Plymouth Rock birds in Table I were passing through the normal puberal change during the course of this experiment, and this fact may account for some of the variations which occurred in testicle size and plasmalogen content of the depot fat of the treated birds.

Table I also includes the results obtained from a group of White Leghorn cockerels eight weeks of age. There appeared to be considerably more plasmalogen in the depot fat of these younger cockerels after estrogen treatment than in the depot fat of the older cockerels (Table I). The younger birds in Table I had not reached the puberal change until after the implantation of the pellet and thus the hormonal balance would be in quite a different state than in the older birds, particularly the cross bred cockerels (Table I). The dosage per kilogram of body weight was greater for White Leghorn birds than for the other two breeds in Table I. This may have had an influence on the plasmalogen content of the abdominal fat.

Fat samples were obtained from a number of estrogen treated and untreated chickens from other laboratories and the results of the plasmalogen test on the depot fat from these samples are recorded in Table II. Plasmalogen was detected in the fat of young males five days after the implantation of the diethylstilbestrol pellet (Table II). Castrated males were checked and there was no sign of any plasmalogen in the depot fat of these birds, although they

TABLE II

ESTROGEN TREATED AND CONTROL CHICKENS TESTED FOR PLASMALOGEN

Breed	No. of birds tested	Sex	Age (wk.)	Dosage (mgm.)	Days on test	Test
White Leghorns	30	Males	12	None	—	All negative
Barred Rocks	5	Capons	12	None	—	All negative
Barred Rocks	5	Capons	12	12	21	All positive
Barred Rocks	14	Males	8	12	5	All positive
Barred Rocks	20	Males	11	12	21	All positive
Barred Rocks	20*	Males	10	12	16	19 positive
Barred Rocks	4	Laying hens	—	None	None	4 positive

* *The one treated bird which failed to give a positive test had large comb, wattles, and testes. Viable sperm were present in the ductus deferens and no pellet residue was recovered from the neck.*

had fatty livers and a large amount of fat in the depot stores. A group of these capons was treated with estrogen and a positive test for plasmalogen was obtained at 21 days after treatment. The body fat of four laying hens was tested and a purple color developed, indicating the presence of plasmalogen.

The results obtained for the depot fat from turkeys are shown in Table III. The young bronze toms were given a subcutaneous injection of a commercial paste containing 10 mgm. of diethylstilbestrol. Biopsies were taken three weeks after estrogen treatment and a deep purple color developed in about three minutes after the Schiff reagent was added to samples of depot fat from treated birds. The one laying hen gave a pronounced plasmalogen test but the control males and non-laying females all gave a negative test. These non-laying hens (Table III) had been laying only a week or two prior to the test.

The sperm test (Table I) was carried out by injecting normal saline into the lumen of the excised ductus deferens, followed by expression of the contents on to a glass slide. The smears were stained according to the method of Casarett (2) except that twice the aniline blue content of the stain was used.

TABLE III

ESTROGEN TREATED AND NON-TREATED TURKEYS TESTED FOR PLASMALOGEN

No. of birds	Age* (wk.)	Sex	Dosage (mgm.)	Days on test	Test
Bronze (small)					
4	14	Males	—	21	All negative
4	14	Males	10	21	+++
6	52	Non-laying hens	—	—	All negative
White Holland (large)					
2	20	Males	—	28	Negative
2	20	Males	12	28	+++
1	52	Laying hen	—	—	+++

* *The age recorded in Table III for the male birds is the age on the day of injection of the diethylstilbestrol.*

Sperm were not detected in the ductus deferens from any of the estrogen treated cockerels, but sperm were present in most of the non-treated birds, indicating that most of these birds had reached puberty at the time of killing.

Discussion

The experimental results here presented suggest that plasmalogen is present in the depot fat of estrogen treated birds and laying hens. There was no detectable plasmalogen in the depot fat of non-laying turkey hens, male turkeys and chickens, or castrated cockerels. The results suggest that this material occurs in the depot fat of birds which are under the influence of either endogenous or exogenous estrogen.

The depot fat of most animals contains very small quantities of phosphorus, suggesting that little or no plasmalogen (an acetyl phosphatide) is present. Preliminary work in this laboratory shows that some phosphorus is present in the depot fat of the estrogenized chicken.

It is well known that chicken fat is particularly susceptible to auto-oxidation, and that this results in the formation of peroxides and aldehydes. These substances will interfere with the test for plasmalogen. Preliminary work on storage samples of both chickens and turkeys suggests that the free aldehydes can be removed without hindering the test. A more complete account of the study of stored samples will be reported in due course.

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THE ANAEROBIC DISSIMILATION OF SEDOHEPTULOSE-2-C^{14} AND SEDOHEPTULOSE-3-C^{14} BY *AEROBACTER AEROGENES*[1]

By A. C. Neish and A. C. Blackwood

Abstract

Sedoheptulose-2-C^{14} and sedoheptulose-3-C^{14} were synthesized from D-ribose and D-erythrose with labelled cyanide by application of the cyanhydrin and the diazomethane syntheses. The sugars were dissimilated under anaerobic conditions by growing cultures of *Aerobacter aerogenes*. The same products were formed as are obtained from glucose. The labelling in the products can be explained to a large extent by the action of transketolase and transaldolase functioning in conjunction with the enzymes of the Embden–Meyerhof scheme, but other reactions appeared to be concerned as well.

Introduction

Experiments on the dissimilation of 1-C^{14} labelled pentoses by *Aerobacter aerogenes* (16) and *Escherichia coli* (6) have given results which can be explained by the conversion of pentose to triose via a heptulose by the combined action of the enzymes, transketolase (8, 18), transaldolase (7), and aldolase. Since *A. aerogenes* will dissimilate sedoheptulose under anaerobic conditions, it is of interest to find if the labelling in the products from the fermentation of labelled sedoheptulose can be explained by the combined action of the above mentioned enyzme systems. This paper reports the results obtained for the anaerobic dissimilation of sedoheptulose-2-C^{14} and sedoheptulose-3-C^{14}.

Experimental

Preparation of Sedoheptulose-2-C^{14} and Sedoheptulose-3-C^{14}

Sedoheptulose-2-C^{14} was synthesized from D-ribose by reaction with $KC^{14}N$ to give cadmium D-altronate-1-C^{14} which was then converted to sedoheptulose by the diazomethane synthesis (21). Sedoheptulose-3-C^{14} was synthesized from D-erythrose by two successive cyanhydrin syntheses, the first with active cyanide, and the second with inactive cyanide, to give cadmium D-altronate-2-C^{14}; this was then converted to sedoheptulose. The techniques used in the cyanhydrin reactions and sodium amalgam reductions were based on those of Isbell *et al.* (9).

D-Erythrose was prepared and reacted with cyanide as described previously (14) except that equimolar amounts of NaOH and $NaHCO_3$, were present during the initial condensation with cyanide. Three or four parts of carrier were used in the isolation of each of the products. Cadmium D-ribonate-1-C^{14} was isolated in 35% yield. Calcium D-arabinate-1-C^{14} (53% yield) was the major product although the ratio of the aldonic acids was somewhat less in favor of the arabonic epimer than obtained previously (14). A higher yield of the ribonic epimer might have been obtained if more alkali were added during the condensation (5).

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D-Ribose-1-C^{14} was prepared by reduction of the lactone with sodium amalgam in an oxalate buffered medium (5). The sirupy ribonic acid was lactonized by heating for 24 hr. at 90° C. under reduced pressure and no attempt was made to isolate a crystalline product prior to reduction. The aldose was obtained in 44% yield as measured by hypoiodite oxidation of an aliquot of the deionized solution. A series of experiments, using various proportions of amalgam, were run. The highest yield obtained was about 55%, based on the cadmium salt. Aldose measurements on the solutions before deionization often gave results corresponding to yields over 90% but this was due to interference from glyoxylic acid, which is formed from oxalic acid during the reduction (14). Deionization of the solution was accomplished by Amberlite IR-120 and IR-4B resins with a loss of not more than 10% of the ribose. The deionized solution of ribose-1-C^{14} was used directly for the cyanhydrin synthesis.

D-Ribose was treated with equimolar amounts of KCN and $NaHCO_3$ to effect synthesis of the altronic and allonic acids. Calcium D-altronate was isolated first (12) (44% yield) and allonic lactone was obtained from the mother liquors (42% yield) after removal of calcium ions by IR-120. Two or three parts of carrier were used to facilitate isolation of these crystalline products.

Sedoheptulose-2-C^{14} was synthesized from 1 gm. (360 microcuries) of cadmium D-altronate-1-C^{14}. Sedoheptulose-3-C^{14} was made from 0.49 gm. (30 μc.) of cadmium D-altronate-2-C^{14}. The cadmium salt was made from an aqueous solution of the calcium salt by removal of calcium ions with IR-120, neutralization of the boiling acid solution with excess cadmium hydroxide, filtration from the excess hydroxide, and evaporation of the filtrate to dryness. The synthesis of sedoheptulose keto-hexaacetate was carried out as previously described (21). This material (0.85 gm. from 0.49 gm. of cadmium salt) was then deacetylated by sodium methylate (5 meq.) in methanol (20 ml.) at room temperature overnight. The methanol was evaporated under reduced pressure, the residue was dissolved in water, deionized, and the solution evaporated by a stream of dry air at room temperature and streaked on two large sheets (57 × 46 cm.) of Whatman 3 MM. paper in the longest dimension. These sheets were developed by the ascending technique using *n*-butanol/pyridine/water (5/2/1.8). Strips cut from the sides of these sheets were developed with alkaline silver nitrate (19) or orcinol (10) reagents. The main band (sedoheptulose) was centered at 11.0 cm. with other bands found at 1.5, 6.5, 15.5, and 19.5 cm. with the silver reagent. These impurities did not react with orcinol. However, a fifth impurity was found at 16.8 cm. which gave a blue color with the orcinol spray. The sedoheptulose band, which was well separated from the others, was cut out from the main sheet and eluted with 30% methanol. The solvent was evaporated at room temperature to give a sirup which was taken up in 2 ml. of water, treated with a little Darco G-60 charcoal, filtered, and evaporated to a colorless sirup. Sedoheptulose prepared in this way was found to be chromatographically

homogeneous in *n*-butanol/pyridine/water, *n*-butanol/ propionic acid/water (4/2/2.6), or *n*-butanol/ethanol/water (4/1/5) when chromatograms were treated with alkaline silver nitrate or orcinol reagents. A chromatogram developed with butanol/ethanol/water was found to show only one radioactive spot on X-ray film (sedoheptulose-2-C^{14}). The synthetic sugar showed the same mobility and color (with orcinol) as the natural sugar isolated from *Sedum spectabile.*

The over-all radiochemical yield of sedoheptulose-2-C^{14}, based on the cyanide, was about 12% while the yield of sedoheptulose-3-C^{14} was only about 0.8%, on the same basis. The low yield in the latter case is partly due to the relatively low yields in the preparation and reduction of the ribonic acid but results mainly from the large number of steps involved.

Preparation of Sedoheptulose from Sedum spectabile

A crude sirup was obtained by a modification of the original method of LaForge and Hudson (11) as follows. Fresh frozen tops of *Sedum spectabile* (9260 gm.) were macerated with water in a Waring blendor to give about 20 liters of a slurry. This was treated with one pound of fresh bakers' yeast for three hours at 25° C. A solution of 300 gm. of neutral lead acetate in 750 ml. of warm water was then added. The mixture was stirred, treated with 10 liters of ethanol, and heated to boiling. The hot slurry was filtered by suction after mixing with about 10 liters of Celite 535. The cake was washed with 8 liters of warm water. The filtrate and washings were concentrated to half volume in an evaporator similar to that described by Bartholomew (1). This concentrate was treated with hydrogen sulphide to remove lead ions, filtered, and then deionized by columns of IR-120 and IR-4B resins each of which contained about one liter of resin. The deionized solution was evaporated to about two liters, treated with charcoal, and filtered. The filtrate was concentrated further to a stiff sirup (133 gm.) in an evaporator similar to that described by Craig (3).

A portion (3.8 gm.) of this crude sirup was dissolved in 15 ml. of water and put on a 4 × 53 cm. Darco G-60/Celite 535 column (20). The column was developed with water and 23 fractions, totalling about three liters, were collected. Each fraction was examined by chromatography on paper with butanol/pyridine/water. The first liter contained several impurities, the next 300 ml. contained sedoheptulose mixed with another unidentified compound, and the last 1700 ml. contained sedoheptulose but no other compounds detectable with alkaline silver nitrate. Evaporation of these last fractions gave 1.6 gm. of a thick colorless sirup which appeared to be pure sedoheptulose by the chromatographic tests applied to the synthetic sedoheptulose (see above). A further check on the purity of the synthetic and natural samples of sedoheptulose was made by comparing the ultraviolet absorption spectrum of the orcinol reaction product (8), with that obtained from crystalline sedoheptulosan. Good agreement was observed between the curves obtained from each sample with a recording spectrophotometer.

Fermentations

The fermentations were run anaerobically with manual pH control in modified Erlenmeyer flasks, as described elsewhere (13). The organism, *Aerobacter aerogenes* PRL R4, was grown aerobically through two transfers in a medium containing 0.5% glucose and 0.5% yeast extract, on a shaker at 30° C. for 24 hr. A small aliquot of the resulting suspension (0.5 ml.) was used to inoculate the fermentation flask. Each fermentation flask contained 1–2 μc. of the synthetic labelled sedoheptulose (accurately measured) and enough inactive natural sedoheptulose (about 50 parts) to make about 1.3 millimoles. The total volume was 20 ml. and contained, in addition to the sugar, phenol red (0.2 mgm.), ammonium dihydrogen phosphate (20 mgm.), dipotassium hydrogen phosphate (20 mgm.), magnesium sulphate heptahydrate (10 mgm.), potassium chloride (10 mgm.), and ferrous sulphate (0.2 mgm.). The salts and sugar were sterilized separately. The initial sedoheptulose content was determined colorimetrically by the orcinol reaction (8) using crystalline sedoheptulosan as the standard. The fermentations were carried out at 30° C. Adjustments of pH were made three or four times each day to approximately pH 7 but the fermentation solution became quite acid overnight.

Separation and Degradation of Products

When the fermentation was finished it was acidified and worked up as described previously (13) to get fractions representing the carbon dioxide, cells, ethanol, 2,3-butanediol, and organic acids. The acids were fractionated by partition chromatography on silicic acid as before (13). Acetic acid was degraded, after converting to sodium acetate and drying, by the method of Phares (17). Succinic acid was crystallized after addition of 100 mgm. of carrier. Carbon dioxide from the succinic acid carboxyl groups was isolated under the same conditions as from the acetic acid carboxyls, but allowing three hours for the reaction with azide. The total activity in the succinic acid was measured by complete combustion of another portion of the same sample and the activity in the methylene groups was then obtained by difference.

The ethanol fraction, in 10 ml. of water, was mixed with 1 ml. of sodium dichromate and 20 ml. of 19 *N* sulphuric acid. After it had stood at room temperature in a glass stoppered flask, for 30 min., the acetic acid formed from the ethanol was isolated by steam distillation, converted to sodium acetate, and degraded.

The 2,3-butanediol fraction (0.2 mM.) was freed of any volatile solvent by steam distillation. It was then refluxed in about 100 ml. of water. An excess of periodic acid (0.4 mM.) was added. The acetaldehyde formed was swept through the reflux condenser by a stream of air and trapped in a bubbler containing 0.5 ml. of *M* sodium dichromate and 20 ml. of 19 *N* sulphuric acid. After 30 min., when the oxidation of the aldehyde to acetic acid was finished,

the contents of the bubbler were transferred to a distillation apparatus with an equal volume of water and the acetic acid isolated by steam distillation and converted to sodium acetate for further degradation.

The lactic acid was oxidized to acetaldehyde and carbon dioxide by permangante (4) with the same apparatus that was used for the oxidation of the 2,3-butanediol. The aldehyde was swept out through the reflux condenser by carbon dioxide-free air and trapped in the acid dichromate solution as described for the diol. The carbon dioxide, formed from the lactic acid carboxyl group, was carried through the dichromate bubbler and trapped in a bead tower charged with an excess of "carbonate-free" sodium hydroxide. The acetic acid was isolated by distillation as described for the diol, converted to the sodium salt, and degraded.

All samples were converted to carbon dioxide and activities and total carbon measured in a proportional counting apparatus similar to that described by Buchanan and Nakao (2). The results, in the absence of added carrier, are expected to have a precision of $\pm$ 1%. In the few cases where carrier was added prior to degradation (lactic acid and succinic acid) the errors are probably greater than this (*ca.* 10%) since errors in determining the small amount of compound initially present are multiplied by the errors in C^{14} determination. The method was sensitive enough to measure 10^{-4} μc. under the conditions used in the present work.

Results

A material balance sheet for the fermentation is given in Table I. The same products were obtained as from glucose. It cannot be concluded that they are produced in different proportions from sedoheptulose than from glucose on the basis of this experiment because of the fluctuations in pH, and the marked effect of pH on the ratio of products (15). Considerable growth

TABLE I

PRODUCTS OF THE ANAEROBIC DISSIMILATION OF SEDOHEPTULOSE BY *Aerobacter aerogenes*

Product	Millimoles	mM./100 mM. of heptulose fermented
Sedoheptulose added	1.30	—
Residual sedoheptulose	0.46	—
Sedoheptulose fermented	0.94	—
Ethanol	0.75	80.0
2,3-Butanediol	0.21	22.4
Acetic acid	0.48	52.1
Formic acid	0.011	1.17
Succinic acid	0.054	5.70
Lactic acid	0.069	7.35
Carbon dioxide	1.66	177
Cell carbon	0.94	100

Fermentation time—5 days. Carbon accounted for—96.8%. CO_2 calculated/CO_2 found = 0.99.

occurred during the five-day fermentation period and the cells formed accounted for almost 15% of the sugar utilized. The carbon recovery was good but it is not possible to calculate an oxidation–reduction balance since hydrogen was not measured and the level of reduction of the cells was unknown. Carbon dioxide production agreed with that calculated from the other products on the assumption that the products were formed via pyruvic acid.

The distribution of carbon-14 in the products is given in Table II. The cell carbon had approximately the activity expected if all carbons of the sedoheptulose molecule contributed equally to the formation of cellular constituents, although carbon-2 may have contributed somewhat more than

TABLE II

DISTRIBUTION OF C^{14} IN PRODUCTS FROM THE DISSIMILATION OF SEDOHEPTULOSE-2-C^{14} AND -3-C^{14}

Product	Relative specific activity*	
	From sedoheptulose-2-C^{14}†	From sedoheptulose-3-C^{14}†
Ethanol		
CH_3—	0.20	11.0
—CH_2OH	39.2	3.3
2,3-Butanediol		
CH_3—	0.17	8.6
—CHOH—	34.6	3.0
Acetic acid		
CH_3—	0.12	8.9
—COOH	32.8	1.7
Lactic acid‡		
CH_3—	—	7.0
—CHOH—	—	0.75
—COOH	—	25.1
Succinic acid‡		
—CH_2—	23.0	10.5
—COOH	14.8	24.1
Carbon dioxide	1.3	27.3
Cell carbon	15.7	13.2

* *Expressed as per cent of the specific activity of the labelled carbon atom in the sedoheptulose fermented.*

† *Specific activity of sugar fermented was 1.73 μc. per mM. for sedoheptulose-2-C^{14} and 0.63 μc. per mM. for sedoheptulose-3-C^{14}.*

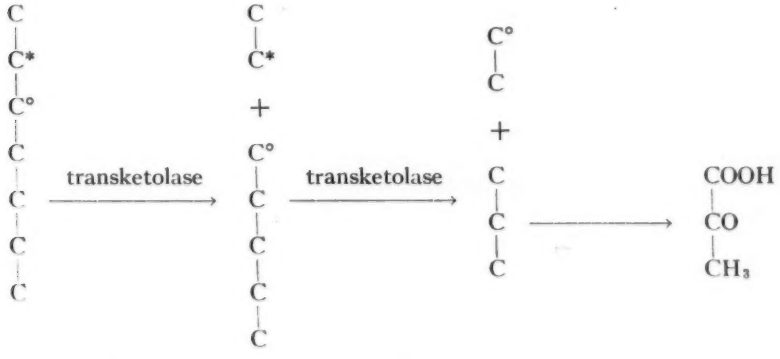
‡ *Ten to twenty parts of carrier added prior to degradation.*

carbon-3. The products from the 2-C^{14} heptulose had the C^{14} still concentrated in one group of all products with the exception of succinic acid. The products from sedoheptulose-3-C^{14} showed appreciable activity in all parts of the molecule, although it was not uniformly distributed. There was a noteworthy difference in the activity of the carbon dioxide from the two fermentations.

The specific activity of the residual sedoheptulose (70% fermented) was measured by combustion of the fermentation solution remaining after removal of all the known products by clarification, distillation, and ether extraction. It was found to be 1.3 times as high as the specific activity of the starting material. This indicates that a small amount of a radioactive, non-fermentable impurity may have been present in the synthetic sedoheptulose even after purification by paper chromatography. If this is true, the figure used for the specific activity of the sedoheptulose was higher than its actual value and as a result the percentage figures in Table II are somewhat lower than their true values.

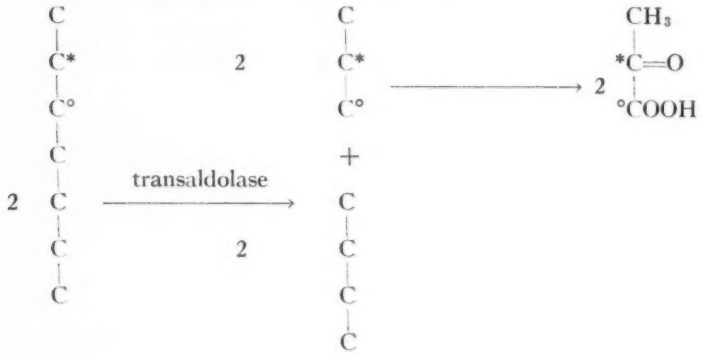
Discussion

The simplest explanation of the anaerobic dissimilation of sedoheptulose that can be made with known enzyme systems is based on the combined action of transketolase and transaldolase to form hexose and triose phosphates, which are then dissimilated further through pyruvate. The fermentation of sedoheptulose by this scheme would start by formation of the 7-phosphate which can serve as a substrate for either transketolase (8, 18) or transaldolase (7). The action of transketolase could produce 2 moles of "active glycolaldehyde" and 1 mole of triose phosphate per mole of sedoheptulose. The result for labelled sedoheptulose would be as follows (the asterisk marks carbon 2 and the circle carbon 3).

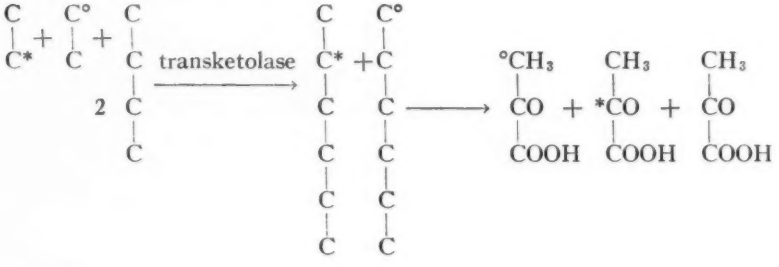


Transaldolase on the other hand, would transfer a dihydroxyacetone group to a triose phosphate giving fructose-6-phosphate leaving D-erythrose-4-phosphate. The dihydroxyacetone group could ultimately be converted to

triose phosphate by the usual Embden–Meyerhof reactions. The over-all result for 2 moles of labelled sedoheptulose would be:



The tetrose-phosphate could combine with the "active glycolaldehyde" formed by transketolase to give fructose-6-phosphate which can be converted to triose-phosphate and finally pyruvate by Embden–Meyerhof reactions.



The over-all result of these reactions is the conversion of 3 moles of sedoheptulose to 7 moles of pyruvate. The pyruvate from sedoheptulose-2-C^{14} would have C^{14} in the carbonyl groups of three-sevenths of the pyruvate molecules formed, and none in any other position. Thus the specific activity of the pyruvate carbonyl carbon would be 3/7 (or 42.8%) of that of carbon-2 of the heptulose. Sedoheptulose-3-C^{14} would be expected to give pyruvate with the methyl group having 1/7 (14.3%) and the carboxyl group 2/7 (28.6%) of the specific activity of the heptulose carbon-3 but with no activity in the carbonyl carbon.

Judging from the labelling actually found (Table II), these reactions play an important role in the fermentation of sedoheptulose but they do not completely explain the results. Considering first the products formed from sedoheptulose-2-C^{14}, the ethanol, and 2,3-butanediol, acetic acid and carbon dioxide appear to have come from pyruvate labelled mainly in the carbonyl group although the activities are a little lower than expected. Furthermore

the activity in the ethanol is significantly higher than that in the diol and acetic acid. The products of the sedoheptulose-3-C^{14} fermentation appear to have been formed from pyruvate labelled in both the carboxyl group and the methyl group, but the activity of the methyl group is quite a bit lower than expected, and some activity is present in the carbonyl group where none was predicted. The activity in the methyl group of ethanol is significantly higher than that in the methyl groups of the other products.

These results suggest that the products are formed mainly from pyruvate, but are also formed by alternate pathways, that do not involve pyruvate. Some of the ethanol may be formed directly from "active glycolaldehyde". Considering the complex nature of a living, growing culture of cells it is not surprising that a simple explanation does not completely fit the results. The labelling in the succinic acid suggests several mechanisms have contributed to its formation. Carbon dioxide fixation is probably involved (13).

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ANTIPHLOGISTIC EFFECT OF TRYPSIN IN NORMAL AND IN ADRENALECTOMIZED RATS[1]

By V. W. Adamkiewicz, W. B. Rice, and J. D. McColl

Abstract

Acute and chronic inflammation were produced in the paws of normal and of adrenalectomized rats by the injection of yeast, egg white, or kaolin. Swelling was measured linearly and volumetrically. Trypsin exerted an antiphlogistic effect on the acute stage of inflammation in both normal and adrenalectomized rats.

Introduction

Trypsin, an enzyme from the pancreas, is known mainly for its proteolytic activity as seen, for example, during the digestion of food, or during the proteolysis of living or necrotic tissue when trypsin is applied topically. Another of its effects is that manifest upon the blood coagulation mechanism: when given in small doses trypsin shortens, and when given in large doses it prolongs the clotting time of blood (2).

Recently an antiphlogistic property of trypsin has been described (7). The parenteral administration of trypsin inhibits certain signs of the inflammatory reaction such as edema and the resultant swelling (6). These observations have been based mainly on clinical data (4, 11). Only a few experiments in animals have been reported (9, 10). We present here observations on the antiphlogistic effect of trypsin as seen during a series of experiments with rats.

Methods

Male and female Sprague–Dawley rats (120–200 gm.) were maintained on Purina Fox Chow and water. Those rats which were bilaterally adrenalectomized received 1% sodium chloride solution in lieu of water.

Crystalline trypsin (National Drug Co., Philadelphia) suspended in sesame oil (Bush, Montreal) or in normal saline, and Parenzyme (Trypsin in Oil—National Drug Co.) were used. The suspension of trypsin in oil, 0.1 ml., was injected intramuscularly into the right or left thigh alternatively. The suspension in saline, 0.05 ml., was given subcutaneously in the back. Parenzyme, 0.01 ml. (0.37 mgm./kgm.), was administered intramuscularly. Pure sesame oil, 0.1 ml., was administered to the control rats in a manner similar to trypsin in oil. Cortisone acetate (Cortone—Merck) was injected subcutaneously in the back at the dose of 15–30 mgm./kgm. (0.07–0.14 ml. of the commercial preparation). Penicillin (Penicillin G – Potassium—Merck), 1 ml. of a saline solution equivalent to 100,000 units, was administered intraperitoneally daily for four days in the course of the chronic experiment.

Acute inflammation was produced in one of the hind paws of the rats by injection into the plantar region of 0.1 ml. of either a filtrate of 20% Brewer's

[1] *Manuscript received December 9, 1954.*
Contribution from the Research Laboratories, Frank W. Horner Limited, Montreal, Canada.

Yeast in water (12), or of fresh egg white diluted 1 : 5 in normal saline (13). Chronic inflammation was produced by the injection of 0.1 ml. of a 10% suspension of kaolin in water. This injection was made in the region of the tibiotarsal articulation (1). After the administration of each irritant, a brief massaging of the area was performed to produce a mild degree of trauma. The resultant swelling was taken as a parameter of the inflammatory edema.

In the case of the yeast filtrate, the circumference of the foot was measured before the injection of the irritant, 30 min. after, and then at hourly intervals. Parenzyme was injected simultaneously with the irritant, then at the 4th, and the 11th hour intervals during the course of the experiment. The difference in the circumference before and after irritation represented the degree of inflammatory swelling.

Where inflammation was produced with egg white and kaolin, the degree of swelling of the foot was determined by an improved volume displacement method ((8), Fig. 1) originally described by Solandt and Best (14). The foot was placed in water containing a wetting agent. The volume of the limb was estimated by measuring the volume of the fluid displaced when the leg was immersed to a known level. This method of measuring gives particularly accurate and reproducible results. In the case of egg white inflammation, measurements by the volume displacement method were made on the same foot (0.5 cm. below the tibiotarsal articulation) prior to the injection of the irritant, 30 min. after injection, and then hourly for six hours. The difference in the volume of the foot before and after irritation was taken as the measure of the inflammatory swelling.

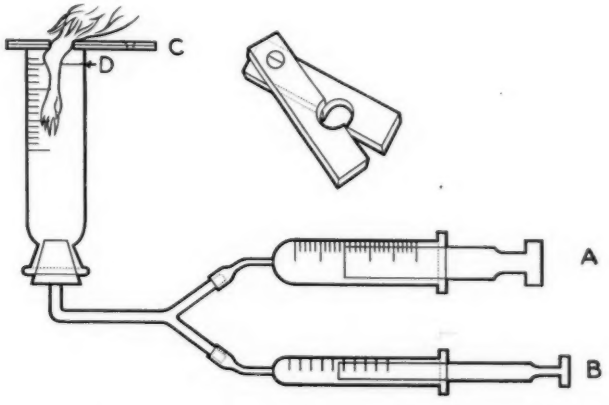

FIG. 1. Apparatus employed for the measurement of the volume of the rat paw during inflammation. The hind leg is secured above the ankle joint with the plastic clamp "*C*". The foot is immersed in the water until the clamp is flush against the top of the cylinder. The fluid displaced by the leg is withdrawn with the syringes "*A*" and "*B*" and then readjusted to the original level "*D*". The volume of water remaining in the syringes represents the volume displaced by the foot. Syringe "*A*" reads to the nearest milliliter and syringe "*B*" to fractions of a milliliter.

In the chronic kaolin experiment, volumetric measurements were made on both hind limbs before the injection of kaolin into one of the paws, three hours following irritation, and then once daily for eight days. The difference between the volume of the normal and inflamed paws was taken as a measure of the edema.

The change in the tibiotarsal articulation itself, during the kaolin experiment, was determined with a caliper by measuring along the anteroposterior and mediolateral axes of the articulation. The sum of these two measurements made on the normal foot was subtracted from the corresponding sum for the inflamed limb, and the difference was taken to represent the degree of swelling. These measurements were done prior to the irritation and daily for eight days after the irritation. The animals were weighed at intervals and autopsied at the conclusion of the experiment. The data were subjected to detailed statistical analysis.

Results

(I) The Effect of Trypsin on a Chronic Type of Inflammation

Fifty male rats (200 gm.) were distributed at random into five groups of 10 animals each. Group I consisted of untreated controls. Group II of controls for treatment with sesame oil, Group III received 30 mgm. of trypsin/kgm. body weight, Group IV received 15 mgm. of cortisone/kgm., and Group V 30 mgm. of trypsin plus 7.5 mgm. of cortisone/kgm. The animals of Groups II, III, IV, and V were pretreated with the drugs for four days. Kaolin inflammation was then produced on the fifth day. The injection of the drugs and the measurements of the foot were continued for an additional eight days. All animals received penicillin prophylactically from the third to the seventh day.

In Groups I and V, one animal died from a pulmonary infection during the experiment. The gain in body weight (Fig. 2) of the untreated controls (Group I) was similar to that of the sesame oil controls (Group II) and to the trypsin treated animals (Group III). The body weight of the animals which had received the full dose of cortisone (Group IV) remained stationary owing to the well known catabolic action of cortisone. The weight of the rats on half the cortisone dose (Group V) increased but to a lesser extent than in the control or the trypsin treated groups.

The increase in the volume of the inflamed foot for the untreated and for the sesame oil controls was of the order of 0.5 ml.; in the trypsin, cortisone, and trypsin plus cortisone groups the increase in volume was considerably lower: 0.35 ml. On the fourth day, this difference was of the same order: the volume in the control groups was 0.24 ml., while in the remaining groups it was 0.09 ml. The marked difference in the amount of swelling between the control and treated groups maintained itself to the end of the experiment (Fig. 3). No essential difference in the inhibition of the swelling was observed between the group which received trypsin plus cortisone and the groups which received trypsin and cortisone separately.

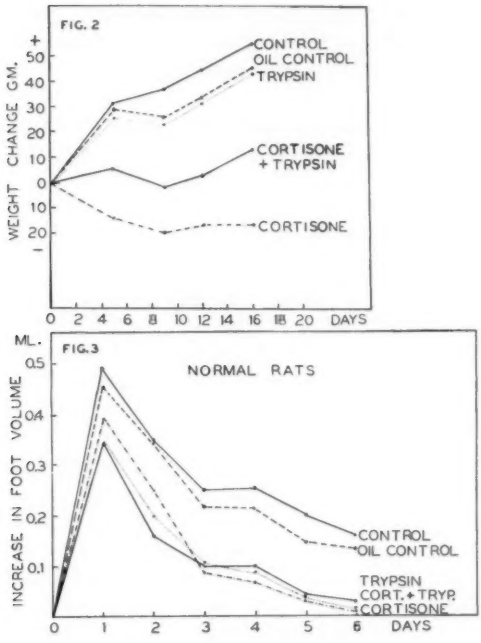

FIG. 2. Change in body weight of normal rats treated with cortisone, trypsin, and cortisone plus trypsin as compared with the control groups.

FIG. 3. Inhibition by trypsin, cortisone, and cortisone plus trypsin, in normal rats, of the peripheral swelling resulting from deposition of kaolin in the region of the tibiotarsal articulation. Inhibition by trypsin is significant ($P = 0.05$) as compared to controls on second and subsequent days; inhibition by cortisone is significant on third and subsequent days.

In this experimental arrangement, and measuring the swelling by the volume displacement method, it appeared that trypsin was as effective as cortisone in inhibiting the peripheral inflammatory edema resulting from a deposition of kaolin in the region of the tibiotarsal articulation.

A somewhat different picture was obtained by the caliper measurements of the tibiotarsal articulation itself (Table I). Here, throughout the course of

TABLE I

MEASUREMENTS OF THE TIBIOTARSAL JOINT IN RATS WITH KAOLIN "ARTHRITIS"

(Figures are mean values in millimeters of the difference in the dimension of the joint before and after irritation during the eight-day experiment.)

Control	Oil control	Trypsin	Cortisone	Cortisone + trypsin
0.35	0.36	0.35	0.29 ($P = > 0.01$)	0.29 ($P = > 0.01$)

the experiment in both groups which had received cortisone, there was a highly statistically significant inhibition of the swelling as would be expected. In the group which had received trypsin alone, no inhibition of swelling could be detected.

At the end of this experiment, an acute egg white inflammation was produced in the remaining normal hind paw of all the rats. These results are described in (II).

(*II*) *The Effect of Long Pretreatment with Trypsin on Acute Inflammation Produced with Egg White*

This effect was studied in the remaining 48 rats from (I). The experimental arrangement was, of course, the same as above, except that the animals had by now been pretreated with the drugs for 12 days and carried a kaolin "arthritis" in one hind paw. On the 13th day, egg white was injected into the remaining hind paw and the resulting swelling was measured by the volume displacement method.

Fig. 4 summarizes these results. The intensity of swelling in both control groups was similar and only the sesame oil control is shown in the figure. The swelling in the trypsin pretreated rats was less than in the control group, and this difference was statistically significant for two hours. Although trypsin did significantly reduce the inflammatory swelling, this inhibition was less than that effected by cortisone. Again, as in (I), no essential difference was observed between the inhibition of swelling in the group receiving trypsin plus cortisone and the groups which had received these two drugs separately.

(*III*) *The Effect of Trypsin on an Acute Inflammation Produced by Yeast Filtrate*

A reverse crossover experimental design was employed. Acute inflammation was produced in two groups of 12 male rats (135 gm.) by injection of yeast filtrate into the plantar region of the hind foot.

The resulting inflammatory edema was inhibited by the administration of trypsin (total dose 1.1 mgm./kgm.). The inhibition was highly significant statistically during the course of the experiment (Fig. 5). The degree of inhibition was greatest (approximately 20% to 30%) from the third to the sixth hour following the irritation.

(*IV*) *Effect of Trypsin on Acute Inflammation Produced by Egg White in Adrenalectomized Rats*

The effect of trypsin was studied in the adrenalectomized animal since the possibility existed that the antiphlogistic effect of trypsin might be mediated through the adrenal gland.

Twenty-two adrenalectomized female rats (125 gm.) were divided into two equal groups. Group I consisted of controls and Group II received two injections of 0.05 ml. of trypsin subcutaneously. The total dose was 10

mgm./kgm. Thirty minutes after the first injection, the volume of the paws was measured in Groups I and II. The second injection of trypsin was then administered to Group II and egg white was injected into the plantar region in both groups. Again, 30 min. later and at hourly intervals, the volume of the paws was measured. The peak of the inflammatory reaction occurred

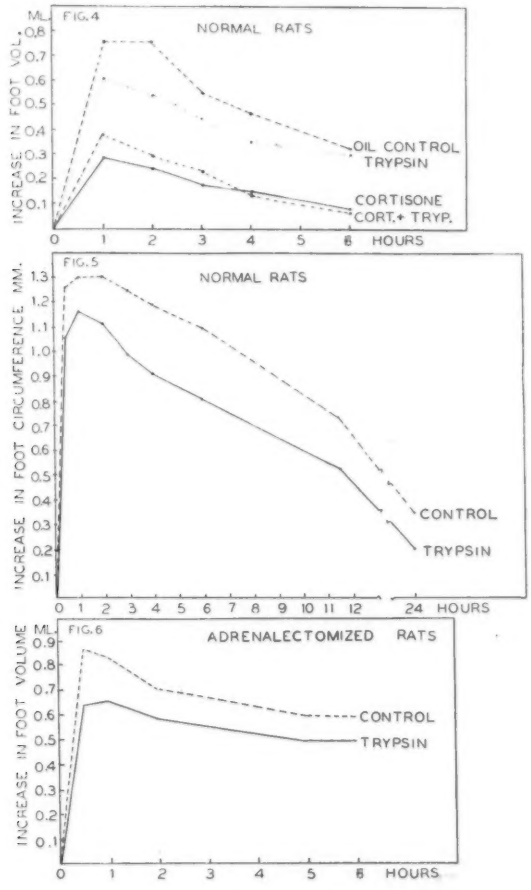

FIG. 4. Inhibition by trypsin, cortisone, and trypsin plus cortisone, in normal rats, of acute inflammation produced by the injection of egg white. Inhibition by cortisone, and cortisone plus trypsin is significant ($P = 0.05$) throughout the experiment; inhibition by trypsin is significant at the first and second hour interval.

FIG. 5. Inhibition by trypsin of acute inflammation produced in the normal rat by injection of yeast filtrate. Response to trypsin is significantly different from control ($P = 0.01$) throughout the experiment.

FIG. 6. Inhibition by trypsin (10 mgm./kgm.) of acute inflammation produced in the adrenalectomized rat by the injection of egg white. Inhibition is significant ($P = 0.01$) as compared to controls at the 30 min., one hour, and two hour interval.

30 min. following the injection of the irritant (Fig. 6). The increased swelling in the control group was 0.85 ml. The swelling in trypsin treated rats was significantly inhibited (23%) and was 0.65 ml. at the same time interval. The inhibition by trypsin was again statistically significant for two hours.

(*V*) *Effect of a Large and a Small Dose of Trypsin on Acute Inflammation in the Adrenalectomized Rat*

Three groups each consisting of 10 female adrenalectomized rats (125 gm.) were used. Group I acted as controls, Group II received a total of 40 mgm. of trypsin/kgm., and Group III a total of 0.4 mgm./kgm. The procedure was the same as in (IV).

No inhibition of inflammation was observed in Group III, indicating that the administered dose was ineffective. In Group II, the inflammation was significantly inhibited to a degree similar to that observed in (IV) where 10 mgm. trypsin/kgm. was administered (i.e., approximately 26–30% inhibition at 30 min. interval). However, the rats of Group II became hypothermic, cyanotic, and comatose and six out of 10 animals died within six hours. At autopsy, hemorrhages in the stomach were consistently noted. The subcutaneous site where trypsin was injected was hemorrhagic and edematous.

Discussion

In this series of experiments on rats, we were able to confirm the observation that crystalline trypsin administered by the subcutaneous or intramuscular routes displays anti-inflammatory properties. This was best seen in acute types of inflammation, such as produced by egg white or yeast filtrate injections in the paw. The volume and duration of swelling in such inflammations were reduced by trypsin.

Trypsin did not appear to exert an antiphlogistic effect on chronic inflammation such as the kaolin "arthritis" in the tibiotarsal joint as measured with a caliper. Here, it only inhibited the peripheral edema which formed in the rat paw below the joint. It may be noted that in clinical trials no inhibition of arthritis was observed following trypsin administration (5).

We wanted to ascertain whether or not the antiphlogistic action of trypsin is mediated through the adrenal gland. Injection of trypsin could cause an alarm reaction which would produce a discharge of endogenous antiphlogistic corticoids. We therefore performed experiments on adrenalectomized rats and we observed that the anti-inflammatory action of trypsin manifested itself in these rats to the same degree as in normal animals. Therefore, the antiphlogistic action of trypsin does not appear to be mediated through the adrenal gland.

The inhibition seemed to be of the same degree whether trypsin was administered by long pretreatment or given simultaneously with the irritant. Increasing the dose of trypsin over a range from 1 mgm. to 40 mgm. did not result in a noticeable increase of the antiphlogistic action. However, a dose

of 0.4 mgm. of trypsin/kgm. was not sufficient to inhibit inflammatory edema produced by egg white in the rat paw. Martin, Brendel, and Beiler (10) have reported that the minimum effective dose observed to inhibit swelling, produced by egg white, is 1 or 2 mgm./kgm.

We suggest that this action of trypsin manifests itself after a certain threshold dose of the enzyme has been administered. By increasing the dose above this threshold, no further significant increase in the anti-inflammatory action is seen.

Administration of trypsin at a dose of 40 mgm./kgm. body weight and in combination with an egg white injection in the paw of adrenalectomized rats, resulted in a 60% mortality with hemorrhages in some of the organs (i.e., stomach). The antiphlogistic effect of such a high dose in the adrenalectomized rat is questionable since here the lack of inflammatory response may be due to shock alone.

The antiphlogistic action of trypsin is, in general, less than that of cortisone. It is also more specific since, in the case of kaolin "arthritis", it appears to be limited to swelling and edema of the foot below the tibiotarsal articulation, while the antiphologistic action of cortisone affects the entire paw. However, the two drugs may not act through the same mechanisms.

Trypsin induces, at the site of injection, signs of acute inflammation: edema, swelling, hyperemia, and hemorrhage. This is due to the proteolytic action of trypsin, since, after injection, the enzyme is concentrated in a small area. The damage may further be increased by the discharge of histamine associated usually with trypsin administration (3).

Trypsin does not inhibit this inflammation resulting from proteolysis of tissue induced by trypsin injection itself. Yet the same trypsin has an antiphlogistic effect on another acute inflammation located somewhere else in the body. Since this action is extra-adrenal, it may be that either trypsin itself or some products of the local proteolysis travel to the second inflammatory focus where they inhibit the reaction, perhaps by affecting the chain of reactions regulating the formation of inflammatory edema.

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A CONSTANT-RATE INDICATOR-INFUSION TECHNIC FOR THE MEASUREMENT OF CENTRAL VASCULAR VOLUME IN MAN[1]

By Dorrance Bowers[2], John T. Shepherd[3], and Earl H. Wood[4]

Abstract

Evans blue dye was injected at a constant rate into the right ventricle or pulmonary artery of 17 subjects in 21 experiments. The consequent arterial dilution patterns were recorded continuously by a cuvette oximeter connected to an indwelling needle in the radial artery. From these dilution patterns the amount and concentration of dye in the intravascular space between the injecting and sampling sites were determined when an equilibrium concentration was attained. From these dimensions the "central vascular volume" was calculated. The values for the central vascular volume thus derived showed agreement with those determined in near-simultaneous estimations by Hamilton's modification of Stewart's method. The values for the "lung blood volumes" by the Newman method in these subjects were systematically smaller than the values for the central vascular volume.

When an indicator is injected into an intravascular compartment, the volume of that compartment can be calculated, if certain assumptions are made, from the indicator-dilution pattern recorded at any of its exits. The injection may be administered as a sudden single "bolus" or as a constant-rate infusion. In the past the "central vascular volume" has generally been determined by the use of the former technic (4). It is the purpose of this report to describe the application of a constant-rate indicator-infusion technic to this measurement.

The calculation of the central vascular volume by the use of this constant-rate indicator-infusion technic is based on the principle that if the amount of indicator in a space and its concentration in that space are known, the volume of the space may be calculated. This principle is employed in the method described by Keith and his associates (6) for the determination of the total blood volume. In order to measure the volume of a subtotal intravascular space, Bradley and his associates (3) modified this method. They calculated the amount of indicator in the space from the amounts of indicator entering and leaving the space. The amount of indicator entering the space was determined from the blood flow through the space (F) and the concentration of indicator in the blood stream entering the space (C_A). Similarly, the amount of indicator leaving the space was calculated from the blood flow and the concentration of indicator in the blood stream leaving the space (C_V).

Dexter and his group (10) have recently applied this principle to the measurement of the central vascular volume of dogs. They determined the amount of indicator entering this space by drawing a continuous blood sample

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from the pulmonary artery, and the amount of indicator leaving the space by a continuous sample from the femoral artery. The central vascular volume was then calculated by the use of the following equation:

$$V = \frac{F\int_0^{T_E} C_A dt - F\int_0^{T_E} C_V dt}{C_E}$$

where T_E is the time from the beginning of the injection to the attainment of equilibrium, and C_E is the equilibrium concentration of indicator.

In the present study it was assumed that at equilibrium (Fig. 1) the concentrations of indicator in the blood stream entering, traversing, and leaving the space were equal. Since the concentration of indicator in the blood stream entering the space is constant,

$$C_A = C_E .$$

$$\text{Accordingly, } V = \frac{F\int_0^{T_E} [(C_E - C_V)dt]}{C_E} . \qquad [1]$$

This equation is theoretically sound. It has been developed independently by Andres and his co-workers (1) for the measurement of the vascular volume of the forearm by the use of a constant-rate indicator-infusion technic. Recently, Meier and Zierler (8) have published mathematical derivations establishing the identity of the volume calculated by this equation and the volume calculated by the Hamilton modification (4) of the Stewart method (13).

In the application of this constant-rate indicator-infusion technic to the measurement of central vascular volume multiple assumptions have been invoked. These assumptions include: (1) that the mixing of blood and indicator is uniform; (2) that the cardiac output is constant; (3) that the time–concentration pattern of indicator at the sampling site is the same at each exit from the space; (4) that the recording apparatus reproduces faithfully the time–concentration pattern of indicator at the sampling site; and (5) that the value chosen for the equilibrium concentration contains no recirculated indicator. On the basis of these assumptions equation [1] may be modified:

$$V = \frac{F \times T_E \times C_E - F\int_0^{T_E} C_V dt}{C_E} . \qquad [2]$$

This is the equation by which the central vascular volumes in man were calculated in the present study. The values derived by this method were compared with those obtained by the use of the sudden single-injection technic. For comparison with these volumes, the "lung blood volumes" in the same subjects were determined by the Newman method (9). A preliminary report of the present study has already been published (2).

CALCULATION OF "CENTRAL" VASCULAR VOLUME

$$\text{VOLUME OF SPACE} = \frac{\text{(Amount of Dye Entering Space)} - \text{(Amount of Dye Leaving Space)}}{\text{(Equilibrium Concentration of Dye in Space)}}$$

$$\text{VOLUME OF SPACE} = \frac{F \times \int_0^{T_E} C_A \, dt - F \times \int_0^{T_E} C_V \, dt}{C_E}$$

$$C_A = C_E$$

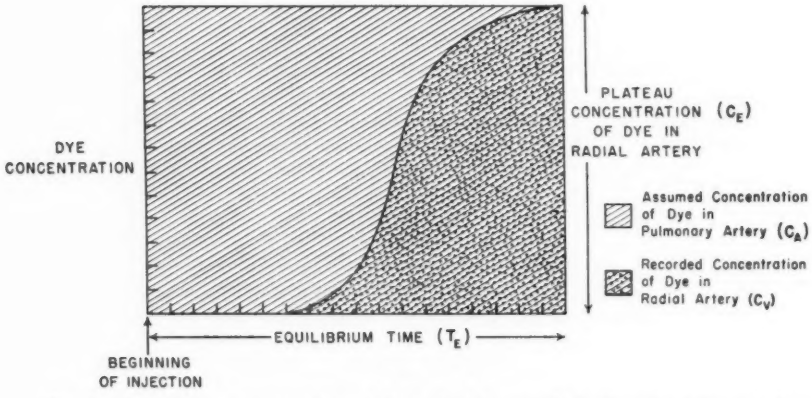

FIG. 1. Schematic representation of the principle on which the calculation of the central vascular volume was based in this study. The amount of indicator entering the space is represented by the rectangular cross-hatched area; the amount of indicator leaving the space is indicated by the stippled area under the sigmoid curve. The amount of indicator remaining in the space at equilibrium may be determined from the difference between these two areas. The amount of indicator in the space at equilibrium and the equilibrium concentration of indicator being known, the central vascular volume may readily be calculated.

Methods

The experiments were designed primarily for the assessment of the constant-rate indicator-infusion technic for determining the cardiac output, and have been described in detail elsewhere (11). The 17 subjects were shown by cardiac catheterization to have no abnormal communication between the pulmonary and systemic circulations. Most of the subjects were studied at rest in the supine position; four were studied at rest and again during moderate exercise.

Under fluoroscopic control the tip of a cardiac catheter was placed in the pulmonary artery or right ventricle. The tip of an indwelling needle was placed in the radial artery; the hub of the needle was connected to a cuvette oximeter. The simultaneous single-and-double-scale method of operating the cuvette oximeter (14) was employed, the deflections of the single-scale and double-scale galvanometers being recorded by a photokymographic assembly previously described (15). The sensitivity was such that 2 mgm. of dye per liter of blood caused a deflection of approximately 1 cm. to be recorded by the single-scale galvanometer on the photographic record. Because the "red"

photocells of the cuvette oximeter are sensitive to changes in the oxygen saturation of the blood, all subjects were studied while they breathed 100% oxygen.

A dye-dilution curve was recorded by the cuvette oximeter following the sudden single injection of Evans blue through the cardiac catheter. Within a short interval of time (average: 9 min.), a dye-dilution curve was recorded by the cuvette oximeter following the injection of dye at a constant rate through the cardiac catheter by a mechanically driven syringe. Approximately 10 min. after the second dye-dilution curve was recorded, a sample of arterial blood was withdrawn. Known amounts of Evans blue were added to aliquots of this sample. These were then run in succession through the cuvette oximeter. The photographic record of the deflections thus produced was used as a calibration to convert to units of concentration, the deflections recorded following the dye injections *in vivo*.

Results

The dye-dilution curves recorded following the sudden single injections of dye were corrected for recirculated dye, and the pulmonary blood flow was determined by the Hamilton method (7). The mean transit time (16) was calculated and added to the corrected appearance time of the dye. From these dimensions, the central vascular volume was calculated by Hamilton's modification of the Stewart method.

From the pulmonary blood flow determined from the dilution curves recorded following the sudden single injections of dye, and the slope of the descending limb of these curves, the "lung blood volume" was calculated by the Newman method (9).

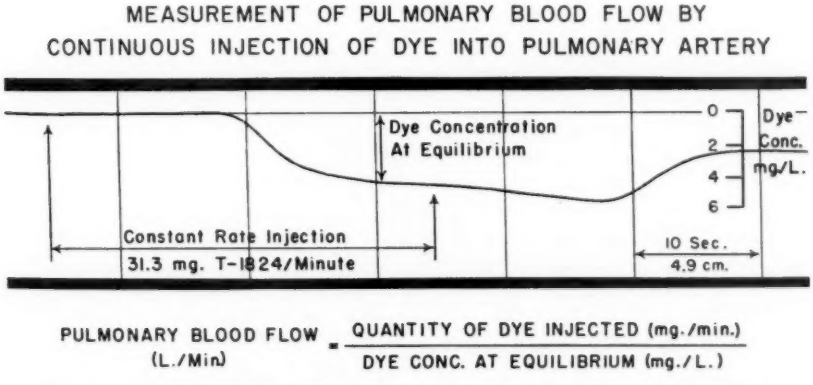

FIG. 2. Photokymographic recording of an arterial dilution curve following the injection of Evans blue dye at a constant rate into the pulmonary artery. The other physiologic variables simultaneously recorded in this experiment have been deleted for the sake of simplicity. The point at which the equilibrium concentration was measured was determined by inspection. The pulmonary blood flow is calculated from the amount of dye injected per minute and the equilibrium concentration of the dye.

From the dilution curves recorded following the constant-rate dye injections the equilibrium concentration (C_E) was determined, and the pulmonary blood flow (F) was calculated (Fig. 2). It has been shown (12) that the pulmonary flow thus calculated is not significantly different in man from the pulmonary flow calculated by the Hamilton method or by the conventional application of the Fick principle. The time from the beginning of the injection to the attainment of equilibrium (T_E) was measured and corrected for the delays in the cardiac catheter and in the cuvette oximeter connected to the radial artery. The mean concentration of dye in the radial-artery blood $\int_0^{T_E} C_V dt$ was determined from the arterial dilution curve in the following manner: the area (in square centimeters) under the dilution curve before the attainment of

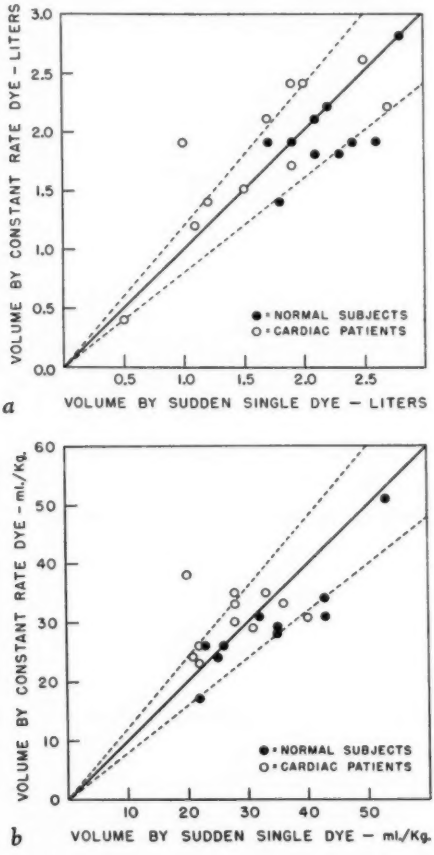

FIG. 3. Comparison of the values for the central vascular volumes determined by the constant rate and the sudden single-injection technics: (*a*) expressed in liters; (*b*) expressed in milliliters per kilogram of body weight. The dotted lines to the sides of the line of identity indicate the $\pm 20\%$ range.

equilibrium was determined by planimetry; this area was divided by the distance (in centimeters) representing the equilibrium time in order to obtain the mean deflection (in centimeters) of the single-scale galvanometer recording the change in the optical density of the radial-artery blood; this mean deflection was then converted to units of concentration (milligrams per liter) by reference to the deflections produced by the known concentrations of dye in the subject's arterial blood. The central vascular volume was then calculated by substituting these parameters in equation [2].

The values obtained by the Hamilton method ranged from 0.6 to 2.8 (mean: 1.9) liters, by the Newman method from 0.2 to 0.7 (mean: 0.4) liters, and by the constant-rate dye-injection method from 0.5 to 2.8 (mean:

TABLE I

COMPARISON OF VALUES FOR CENTRAL VASCULAR VOLUME DETERMINED BY THE CONSTANT-RATE DYE-INFUSION TECHNIC AND BY HAMILTON'S MODIFICATION OF STEWART'S METHOD WITH "LUNG BLOOD VOLUME" VALUES DETERMINED BY THE NEWMAN METHOD

Subject	Age, years, and sex	Weight, kgm.	Cardiovascular status	Condition	Central vascular volume*		"Lung blood volume",* Newman method
					Constant-rate dye method	Sudden single dye method	
1	26 M	73.9	Normal	Rest	26	23	5
2	17 M	77.3	Normal	Rest	24	25	4
3	29 M	68.8	Normal	Rest	28	35	7
4	27 M	54.5	Normal	Rest	34	43	7
5	28 M	82.8	Normal	Rest	17	22	4
6	27 M	60.5	Normal	Rest	29	35	6
3	29 M	68.8	Normal	Exercise	31	32	6
4	27 M	54.5	Normal	Exercise	51	53	8
5	28 M	82.8	Normal	Exercise	25	25	5
6	27 M	60.5	Normal	Exercise	31	43	11
7	20 M	69.1	Postoperative coarctation of aorta	Rest	31	40	6
8	6 M	16.0	Postoperative closure of aortico-pulmonary window	Anesthesia	33	36	10
9	46 F	52.0	Postoperative mitral stenosis	Rest	23	22	6
10	25 F	44.1	Mitral stenosis	Rest	35	33	12
11	45 F	49.7	Mitral stenosis	Rest	38	20	5
12	45 F	58.9	Mitral stenosis	Rest	24	21	6
13	20 M	69.1	Coarctation of aorta	Rest	35	28	4
14	34 M	60.0	Coarctation of aorta	Rest	29	31	5
15	28 M	71.4	Aortic regurgitation	Rest	33	28	7
16	19 M	88.2	Essential hypertension	Rest	30	28	5
17	66 M	78.6	Pulmonary fibrosis	Rest	26	22	4

** The values for these volumes are expressed in milliliters per kilogram of body weight.*

1.9) liters. These volumes expressed in milliliters per kilogram of body weight were 30.7 (S.D. 8.8), 6.3 (S.D. 2.3), and 30.1 (S.D. 6.9) respectively (see Table I). There was no significant difference between the results obtained by the constant-rate dye-injection and the Hamilton methods (Fig. 3). The Newman values were uniformly lowest. The values for these volumes in the normal subjects and in the patients with cardiovascular disease overlapped.

Comment

In their present application both the constant-rate dye-injection method and the Hamilton method measured the volume of a space which included the pulmonary intravascular space, the chambers on the left side of the heart, and the arterial tree peripherally in all directions to a distance corresponding to a time interval equal to the time required for blood to travel from the left ventricle to the radial artery. For the sake of convenience this volume has been called the "central vascular volume".

The value of the determination of the central vascular volume is limited when a peripheral artery is chosen for the distal sampling site. Physiologic or pathologic changes in the volume of the pulmonary vascular tree may be obscured by the unknown extrapulmonary fraction of the central vascular volume. If the left atrium or ascending aorta were employed as the distal sampling site, this fraction would be reduced, and a closer approximation of the pulmonary vascular volume would theoretically be possible.

A reliable method for the measurement of the pulmonary vascular volume would be of obvious value. In the only method presently available, the Newman method, this volume is calculated from the ratio of the pulmonary blood flow to the slope of the logarithms of declining dye concentrations following sudden single injections. Newman's group postulated that this slope is determined by the washout of dye from the largest volume in the central vascular system, presumably the lungs. Hetzel, Swan, and Wood (5), however, have demonstrated that this slope is influenced by the site of dye injection, thus weakening the premise on which the Newman method is based.

Because this constant-rate infusion technic may be modified for the measurement of other intravascular volumes or for the closer approximation of the pulmonary vascular volume, the assumptions on which it is based have been made explicit. It should be emphasized that some of these assumptions are common to the constant-rate infusion technic and to Hamilton's modification of the Stewart method. Accordingly, despite the apparent agreement in this study between the values obtained by the two methods, the reliability of the methods and the significance of the values calculated by their use should remain sub judice until the potential errors introduced by these assumptions are evaluated.

It may be concluded that the constant-rate indicator-infusion technic is a practical method for the measurement of the central vascular volume and that the values determined by this method showed agreement with those obtained by Hamilton's modification of Stewart's method.

Summary

1. A formula has been derived for the calculation of the volume of an intravascular compartment. If V is the volume of the space, F is the flow through the space, T_E is the time from the beginning of the injection of indicator to the attainment of equilibrium concentration, C_E is the equilibrium concentration, and $\int_0^{T_E} C_V dt$ is the mean concentration of indicator leaving the space, then

$$V = \frac{F\int_0^{T_E}[(C_E - C_V)dt]}{C_E} .$$

2. The parameters required for insertion in this formula may be measured from the indicator-dilution curve recorded at an exit from the space following the injection of indicator at a constant rate into the blood stream entering the space.

3. This technic has been applied to the determination of central vascular volume in man, and the results compared with near-simultaneous determinations of related volumes by the Hamilton and Newman technics.

4. The values for the volumes determined by the constant-rate dye-injection method showed agreement with those by the Hamilton method. Both of these values were greater than the values for the "lung blood volumes" calculated by the Newman technic.

5. Because some of the same assumptions are common to the constant-rate infusion method and to the Hamilton method for calculating the central vascular volume, the agreement observed between the values determined by the two methods does not establish the reliability of either method.

Acknowledgments

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CARDIOVASCULAR RESPONSES IN DOGS TO INTRAVENOUS INFUSIONS OF WHOLE BLOOD, PLASMA, AND PLASMA FOLLOWED BY PACKED ERYTHROCYTES[1]

By F. A. Sunahara, J. D. Hatcher, L. Beck, and C. W. Gowdey

Abstract

The effects of intravenous infusions of large volumes of blood or of plasma followed by packed erythrocytes were studied in anesthetized normal dogs. During plasma infusion the right auricular pressure (RAP) and cardiac output increased as the hematocrit decreased. Blood infusion caused a rise in RAP but was, in most cases, not accompanied by an increased output. It is concluded that, although the blood volume and RAP may be important in the regulation of cardiac output, they are not under all conditions the controlling factors. The relative oxygen-carrying capacity of the blood appears to be more important in the cardiovascular adjustments to hypervolemia.

Introduction

In the previous paper (8) it was shown that infusion of gum acacia solution increased the cardiac output and right auricular pressure (RAP) in dogs. Other investigators have reported that intravenous infusions of blood, plasma, or plasma substitutes produced an increase in the cardiac output in normal man (1, 4) and in both open-chested and closed-chested dogs (3, 6, 8, 9, 11, 14, 16, 19, 25, 27). Warren *et al.* (24) failed to find a correlation between RAP and cardiac output with intravenous saline or albumin infusions. Cournand *et al.* (5) have shown that in shocked patients saline or albumin increased the cardiac output above normal levels, but whole blood increased cardiac output only to normal levels.

Preliminary experiments indicated that although infusions of acacia increased both right auricular pressure and cardiac output, whole blood infusions caused the RAP to rise but not the cardiac output (7). To elucidate the factors affecting cardiac output the effects of infusions of blood, plasma, and plasma followed by packed erythrocytes have been studied in anesthetized intact dogs.

Method

The methods employed in this study were similar to those described previously (8). The direct Fick method was used for cardiac output measurements. The arterial and right auricular pressures were determined by mercury and saline manometers respectively. Blood obtained from one or more anesthetized heparinized dogs was cross-matched with that of the recipient dog. After a suitable control period a constant intravenous infusion of the blood or plasma was begun and continued until the death of the animal, or until the volume infused was greater than the dog's original calculated blood volume. For the plasma and packed-erythrocyte infusion experiments whole blood was centrifuged, and the plasma and red corpuscles carefully

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separated to prevent hemolysis. In the first whole-blood series the ordinary gravity intravenous set-up was used. The rate of infusion was measured continuously by a specially-built drop recorder using a relay and impulse counter. In other experiments a Dale-Schuster pump was employed, and periodic readings of the volume infused were made. The packed erythrocytes were injected from a 50 ml. syringe at the same rate as the plasma had been infused (0.77 ml. per kgm. per min.).

Results

Three series of experiments were performed. In Series "*A*" five dogs were infused with whole blood at an average rate of 0.6 ± 0.11 ml./kgm./min. (S.D.) (dogs 45 to 49). Table I–*A* shows the effects of infusing whole blood at this moderate rate. The RAP increased gradually as infusion continued, reaching an average of 180 mm. saline at the end of the infusion. In spite of the increased filling pressure, the cardiac output, on the average, did not change significantly from control levels. Mean brachial arterial blood pressures showed no consistent changes during or following the infusions.

In Series "*B*" blood was infused at a faster rate, 2 to 3 ml./kgm./min. Table I-*B* summarizes the results of this series, and Fig. 1 shows a typical example. The results were similar to those of Series "*A*", but generally the RAP increased more rapidly. Adrenaline was infused both before and after the blood infusion to test the ability of the heart to increase its output at these times. Five γ/kgm./min. was found adequate to increase the cardiac output. The cardiac output, after the blood infusion, increased in two of the six dogs of this series. This increase in cardiac output could not be correlated with either the rate of infusion or the rise in RAP; in the remaining experiments where the rates of infusions were similar and the increases in the RAP comparable, no increase in the cardiac output was observed.

Hemoconcentration was observed in all of the whole-blood infusion studies. The maximum values usually occurred at the termination of the infusion. The mean hematocrit increase was 15% ± 4.0 (S.D.). In spite of hemoconcentration, the average calculated total peripheral resistance did not alter significantly. In Fig. 2, the means of the various indices (± S.E. mean) of both Series "*A*" and "*B*" are plotted against the volume of blood infused expressed as a percentage of body weight.

The responses to plasma infusion were similar to those to acacia infusion in most respects (8); the RAP and the cardiac output increased with a concomitant decrease in the hematocrit. The calculated total peripheral resistance decreased during the plasma infusion, Fig. 3, Table I (Series "*C*"). When packed erythrocytes (hematocrit 84%) were infused following plasma, the hematocrit immediately returned to control values or above. The RAP continued to increase at the same rate as during the plasma infusion, but the average decrease of 85% in cardiac output was significant ($P = 0.02$–0.01).

Post-mortem gross examination revealed a greatly dilated heart and systemic veins. Pulmonary edema was frequently evident after blood infusion. The liver and spleen were invariably engorged.

TABLE I

Dog No. wt., sex	Time (min.) after infusion started	Cardiac output (l./min.)	Art. O_2 (vol.%)	Mean art. B.P. (mm. Hg)	Right auric. press. (mm.H_2O)	Total per. resis.*** (dynes sec. cm.$^{-5}$)	Heart rate (beats/ min.)	Hct. (%)	Vol. inf. (ml.)
				SERIES "*A*"					
45	−35*	2.75	19.20	106	17	3050	128	38.5	0
13 kgm.	0	Infusion begun							
Female	45	1.70	20.15	116	120	5020	140	45.5	400
	115	2.45	22.45	106	140	3090	172	61.0	600
46	−33*	2.70	15.55	136	45	3940	140	36	0
16 kgm.	− 3*	2.10	15.40	140	50	5180	130	35	0
Female	0	Infusion begun							
	17	2.40	15.60	136	95	4310	128	35	280
	42	2.15	17.40	126	56	4540	140	39	800
	67	2.00	18.85	128	105	4800	160	43	1100
	97	1.90	19.75	134	136	5200	144	47	1400
	124	1.80	19.00	144	222	5690	136	46	1850
47	−27*	1.85	14.70	140	60	5930	160	36	0
16 kgm.	− 2*	1.70	15.05	144	80	6630	150	36	0
Male	0	Infusion begun							
	25	2.00	17.03	176	84	6930	172	40	350
	50	2.15	21.35	130	92	4680	180	52	675
	95	1.25	23.70	140	110	8610	152	55	1000
	145	1.90	22.00	140	145	5720	160	57.5	1210
48	−40*	2.35	16.40	118	10	3980	120	37.5	0
15.5 kgm.	− 5*	2.35	16.40	116	38	3860	128	39.0	0
Male	0	Infusion begun							
	55	3.35	19.60	110	27	2580	124	47.0	519
	90	2.50	22.10	135	87	4150	130	50.0	787
	130	2.60	22.60	132	125	3780	134	53.0	951
	175	2.55	21.50	124	176	3490	150	56.0	1372
	225	2.65	24.50	114	200	2990	140	57.0	1801
49	−20*	2.75	23.00	107	30	3000		57.0	0
23 kgm.	0	Infusion begun							
Male	37	2.15	23.00	110	124	3850		53.0	291
	95	1.65	23.20	118	225	4760		57.0	971
	150	2.70	19.90	115	242	2930		68.0	1590
	170	2.40	18.40	110	244	3040		68.0	1832

* *Control measurements.*

** *After sciatic stimulation (20 volts, 10 μsec., frequency 400 per min.).*

*** *Total peripheral resistance (in dynes sec. cm.$^{-5}$)* $= \dfrac{\text{Arterial blood pressure} - \text{right auricular pressure (mm. Hg)} \times 1332}{\text{Cardiac output (cc./sec.)}}$

TABLE I—(*Continued*)

Dog No. wt., sex	Time (min.) after infusion started	Cardiac output (l./min.)	Art. O_2 (vol.%)	Mean art. B.P. (mm. Hg)	Right auric. press. (mm.H_2O)	Total per. resis.*** (dynes sec. $cm.^{-5}$)	Heart rate (beats/ min.)	Hct. (%)	Vol. inf. (ml.)
				SERIES "*B*"					
59	−60*	1.85	20.35	100	28	4220		45.5	0
17 kgm.	− 7*	1.90	21.20	125	31	5150		47.0	0
Female	0	Infusion begun							
	28	2.55	23.30	130	240	3740		58.0	1200
	53	3.90	25.00	127	165	2360		66.5	2400
	78	3.40	26.00	125	37	2880		68.0	
	128**	2.90	26.50	127	143	3160		69.0	
60	−85*	2.05	24.30	107	40	4100		53.0	0
17 kgm.	−30*	1.90	22.90	105	26	4330		54.0	0
Male	0	Infusion begun							
	24	1.90	23.40	105	165	3920		51.0	900
	57	2.70	24.20	108	216	2730		54.0	2100
	87	2.55	22.20	102	75	2940		59.5	
	117**	2.45	22.70	94	112	2820		60.0	
	150	2.75	22.40	86	77	2340		59.0	
	180**	2.92	22.10	86	77	2200		62.0	
61	−75*	1.80	18.80	130	25	5700		43.5	0
16 kgm.	−15*	1.65	18.40	128	64	6000		43.0	0
Male	0	Infusion begun							
	15	1.20	19.05	145	182	8750		49.0	500
	31	1.30	20.30	143	338	7240		54.5	1300
	47	1.55	14.10	152	346	6460		64.0	2000
62	−180*	2.50	20.10	120	5	3850	150	43.0	0
16.5 kgm.	− 30*	2.00	20.60	126	15	4990	160	46.0	0
Male	0	Infusion begun							
	15	2.00	19.90	125	40	4900	180	46.0	1000
	31	2.20	20.30	128	60	4150	185	53.0	2150
63	− 20*	2.25	23.60	105	17	3800	160	50.0	0
19 kgm.	5*	2.59	23.60	109	16	3340	156	50.0	0
Female	0	Infusion begun							
	19	1.20	21.60	104	12	6820	150	47.5	900
	67	2.95	24.30	113	70	2930	140	54.0	2240
64	−120*	3.00	21.20	112	16	3500	160	52.0	0
17 kgm.	− 75*	3.30	22.20	122	9	2930	156	53.0	0
Male		5 gamma per min. adrenaline infusion for 20 min.							
	− 55	5.40	22.50	116	53	1660	168	50.0	0
	− 10*	2.70	21.00	114	35	3290	150	46.0	0
	0	Infusion begun							
	10	3.20	21.60	108	153	2420	160	49.5	500
	19	2.95	22.70	118	234	2740	156	47.0	1000
	39	2.86	22.80	128	328	2930	164	51.5	1990
	40	5 gamma per min. adrenaline infusion for 15 min.							
	54	3.78	26.60	144	208	2730	170	58.0	

* *Control measurements.*

** *After sciatic stimulation (20 volts, 10μsec., frequency 400 per min.).*

*** *Total peripheral resistance (in dynes sec. $cm.^{-5}$)* $= \dfrac{\text{Arterial blood pressure (mm. Hg)} - \text{right auricular pressure (mm. Hg)} \times 1332}{\text{Cardiac output (cc./sec.)}}$

TABLE I—(*Concluded*)

Dog No. wt., sex	Time (min.) after infusion started	Cardiac output (l./min.)	Art. O_2 (vol.%)	Mean art. B.P. (mm. Hg)	Right auric. press. (mm.H_2O)	Total per. resis.*** (dynes sec. cm.$^{-5}$)	Heart rate (beats/ min.)	Hct. (%)	Vol. inf. (ml.)
				SERIES "C"					
72	− 70*	1.80	20.30	122	5	5370		46	0
14 kgm.	− 45*	1.55	19.65	120	70	6500		44	0
Male	− 15*	1.70	19.80	108	−15	5120		45	0
	0	Plasma infusion begun (hct. 0.3%)							
	15	2.00	16.40	106	77	4000		38	315
	30	3.90	16.50	94	12	1910		39	545
	52	Packed R.B.C.—infusion begun (hct. 86%)							0
	62	3.25	21.50	90	90	2050		50	299
	84	2.70	24.70	116	116	3170		58	811
	90	Infusion off							
	157	1.25	25.60	122	130	7180		62	
74	−100*	1.25	23.60	134	−10	8450		52	0
21.1 kgm.	− 40*	1.55	22.50	144	10	7390		51	0
Female	0	Plasma infusion begun (hct. 0.6%)							
	30	3.40	19.60	156	105	3500		44	435
	55	4.05	18.20	144	40	2790		43.5	988
	60	Packed R.B.C. begun (hct. 80%)							0
	80	2.25	20.50	130	93	4370		46.5	440
	102			136	180			58	940
48	− 80*	3.75	20.30	148	− 5	3160	180	46	0
18.5 kgm.	− 15*	2.80	20.20	160	−10	4520	168	46	0
Male	0	Plasma infusion begun							0
	20	4.25	17.30	144	13	2690	164	39.5	705
	40	3.55	16.50	143	14	3220	172	38	880
	45	Packed R.B.C.—begun							0
	65	2.80	19.40	150	21	4210	148	45	300
	85	3.20	22.90	136	10	3390	148	55	600
49	−125*	2.60	24.20	140	−10	4310	176	53.0	0
18 kgm.	− 10*	2.10	23.70	130	15	4920	164	52.0	0
Female	0	Infusion begun (hct. 0.5%)							
	35	2.15	19.40	154	85	5500	195	43.0	490
	80	3.05	15.90	132	33	3400	204	34.0	1080
	110	5.40	14.70	124	133	1690	200	31.0	1560
	112	Infusion of R.B.C. begun (hct. 84.0%)						84.0	0
	130	2.80	21.20	130	178	3340	196	37.0	320
	146	2.95	22.40	116	195	2770	190	52.0	560

* *Control measurements.*

** *After sciatic stimulation (20 volts, 10 μsec., frequency 400 per min.).*

*** *Total peripheral resistance (in dynes sec. cm.*$^{-5}$) $= \dfrac{\text{Arterial blood pressure (mm. Hg)} - \text{right auricular pressure (mm. Hg)} \times 1332}{\text{Cardiac output (cc./sec.)}}$

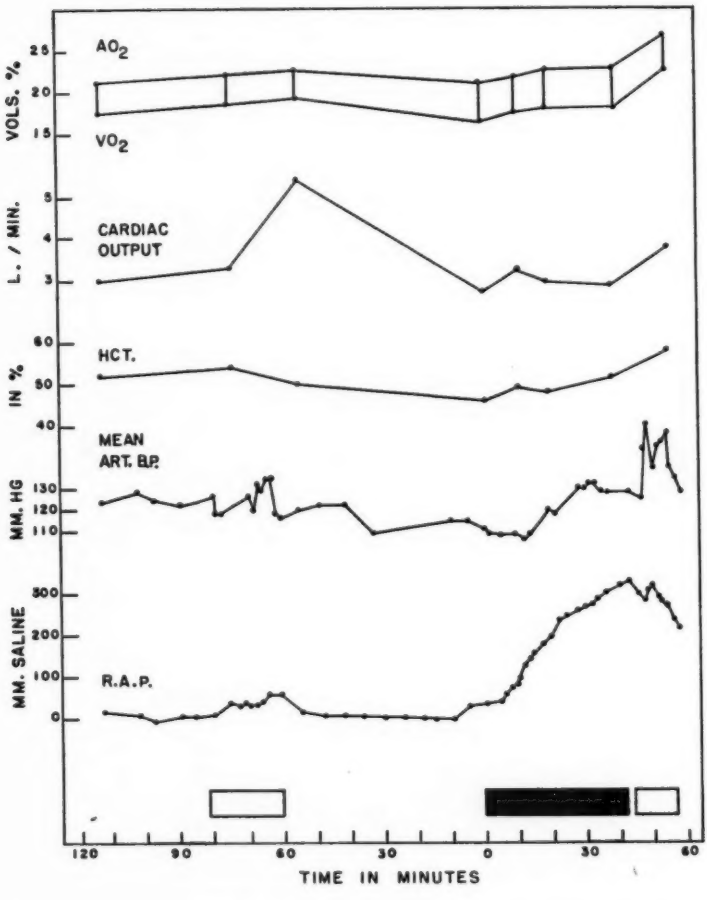

FIG. 1. Cardiovascular effects of whole-blood infusion; dog 64, 17 kgm. male. From above downwards: AO_2, VO_2—arterial and venous blood oxygen in volumes per cent; cardiac output in liters per minute; hct.—hematocrit in per cent; mean art. B. P.—mean arterial blood pressure in mm. Hg; RAP—right auricular pressure in mm. saline; the blank rectangles represent the times at which adrenaline was infused at the rate of 5 γ per min. per kgm. body weight; the solid area represents time of blood infusion, 2.9 ml. per min. per kgm.

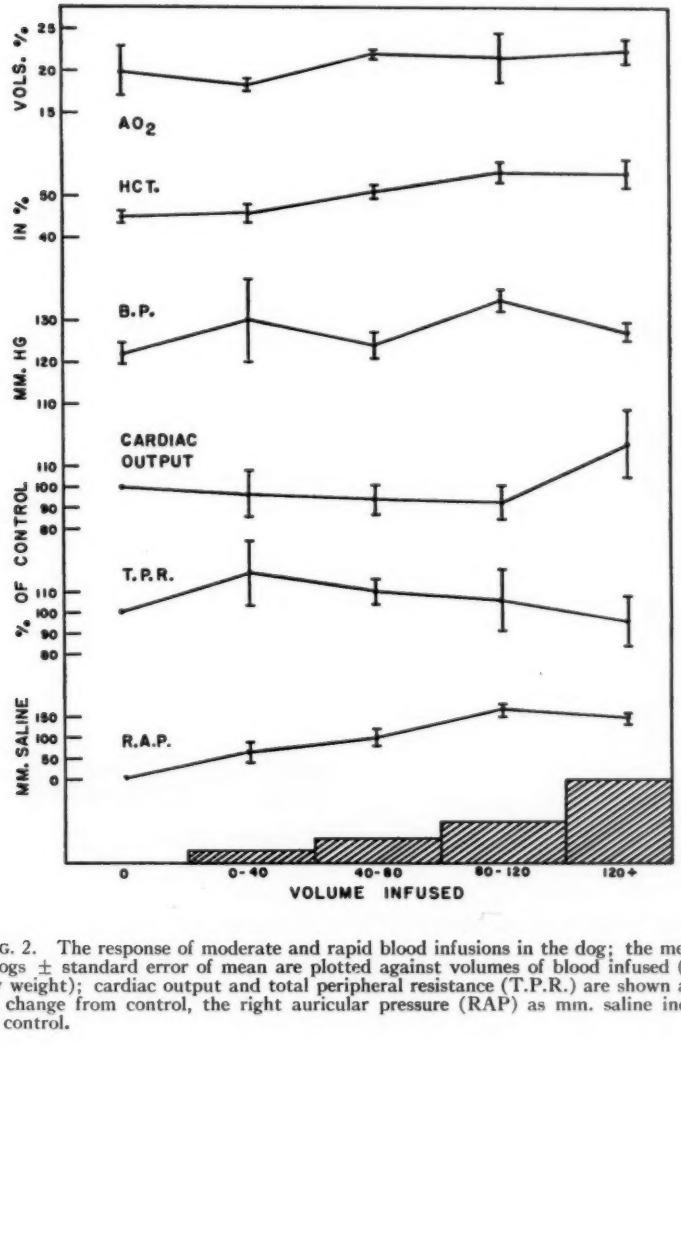

FIG. 2. The response of moderate and rapid blood infusions in the dog; the mean of 11 dogs ± standard error of mean are plotted against volumes of blood infused (% of body weight); cardiac output and total peripheral resistance (T.P.R.) are shown as per cent change from control, the right auricular pressure (RAP) as mm. saline increase over control.

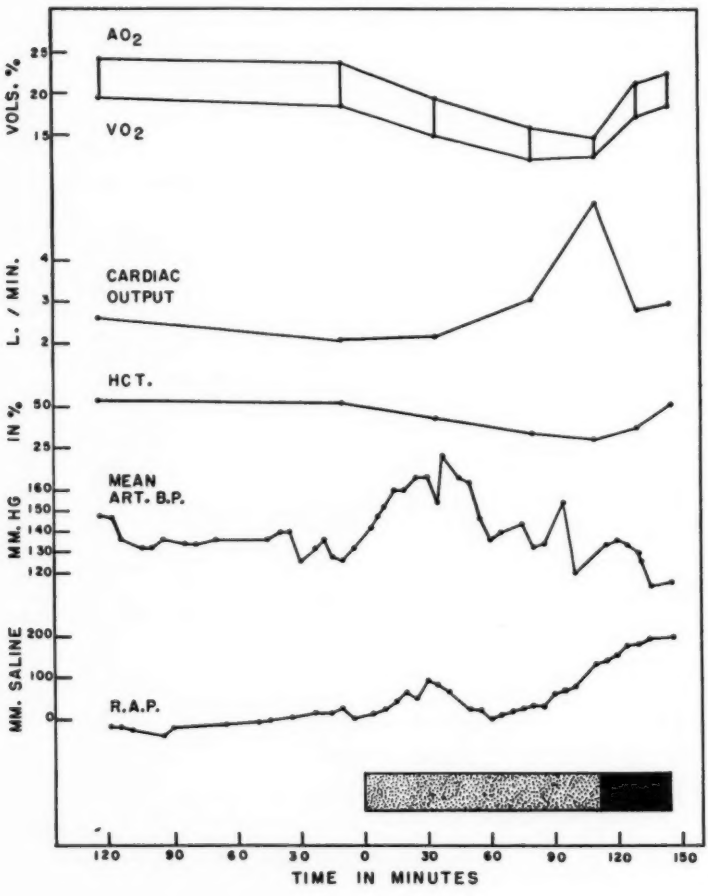

FIG. 3. Cardiovascular effects of infusion of plasma followed by packed erythrocytes; dog 76, 18 kgm. female. The units are similar to those of Fig. 1. Stippled area represents plasma infusion at 0.8 ml. per min. per kgm. The solid area represents packed erythrocyte infusion at 1.1 ml. per min. per kgm.

Discussion

Landis (12) has discussed the many factors which may influence the response of venous pressure to infusion. He found that usually 0.9 to 3.6 ml./kgm./min. of blood had to be administered in order to maintain a venous pressure of 50 to 200 mm. of saline in the dog. Altschule (1) found that in some patients an intravenous infusion of saline or glucose failed to increase the venous pressure, while in other patients the same rate of infusion caused a rapid rise in the venous pressure.

In the present study it was evident that failure of the right auricular pressure to increase steadily was not due to changes in the rate of plasma or blood infusion for this was kept constant. It was probably caused by rhythmic changes in venous tone which were characteristic of the individual animal. This is borne out by several experiments in which the RAP was observed to increase in a saw-toothed manner. Cardiac output measured at the crest or base of these pressure fluctuations showed no consistent relation to the coincident RAP. The infusion of blood caused increases in the RAP of the same magnitude as in the acacia experiments (8). Although the cardiac output invariably increased in the acacia and plasma infusion experiments, in 9 out of 12 blood infusion experiments the cardiac output did not increase significantly. In one of the remaining three dogs there was a transient rise in output (Table I-*B*, No. 48), but before the infusion had been completed the output had returned to control levels. In the other two dogs (Nos. 59, 60) the maximum outputs were 200% and 140% of control; the reason for this could not be determined.

The coefficient of correlation between cardiac output and hematocrit obtained from all the data of the blood infusion experiments was -0.35 ($P = 0.02$–0.01). There was, however, no correlation between cardiac output and RAP ($r = +0.001$; $P > 0.1$). Although the correlation of cardiac output and hematocrit was not as great here as in the acacia infusion experiments ($r = 0.53$, $P < 0.01$) (8), it was evident in the blood infusion experiments that the hematocrit played a role in the regulation of the output, whereas RAP had little influence.

Ferguson *et al.* (6) reported that in closed-chested dogs there was a point to point correlation between end-diastolic pressure, stroke work, and cardiac output in 13 of 16 dogs after a rapid blood infusion; with moderate infusions 5 of 10 dogs showed a correlation between these indices. Sarnoff and Berglund (19) found that there was a correlation between atrial pressure and stroke work on the same side of the heart; however, the descending limb of the Frank-Starling curve (16) did not occur. These results are in part at variance with our findings. Calculations in Series "*A*" showed no significant increase in the average stroke work of the left ventricle, and since there is a direct relationship between left and right auricular pressures (8, 23), it can reasonably be concluded that there was no correlation between filling pressure and stroke work.

The depression of the cardiovascular system caused by the anesthetic present in the administered blood can hardly account for the failure to elicit increased outputs. The donor dogs were given just sufficient anesthetic (ether or pentobarbital) to allow cannulation of a femoral artery, and bleeding was not begun until the anesthetic had almost worn off. Secondly, during and after the blood infusion the recipient dog still required maintenance doses of anesthetic.

In contrast to the plasma infusion experiments, in some of the blood infusion experiments there was a transient increase in the calculated total peripheral resistance. In order to demonstrate that the absence of an increased cardiac output after the infusion of the whole blood was not due to the inability of the heart to work against this high resistance, attempts were made to increase the cardiac output. When the animal was made to exercise by stimulation of the peripheral ends of the cut sciatic nerves (dogs 59 and 60), it apparently satisfied the demand for more oxygen by increasing the percentage utilization of blood oxygen rather than by increasing the cardiac output. A slow infusion of adrenaline following the blood infusion caused a 38% increase in cardiac output in spite of the simultaneous rise in arterial blood pressure from 128 to 144 mm. Hg (dog 64). Thus it was shown that the heart was capable of increasing its output against the increased peripheral resistance.

It had been demonstrated that, although there was some correlation between RAP and the cardiac output in acacia hypervolemia, the correlation of hematocrit and cardiac output was better and more consistent, and thus probably of more importance (8). Observations in open-chested animals were similar (9). In the present study there was little or no increase in cardiac output in spite of the continuing blood infusion and the rising RAP. Moreover, the administration of packed erythrocytes after plasma infusion caused a prompt fall in cardiac output which was coincident with the increase in hematocrit.

In our experiments the diastolic size was not measured. Meek and Eyster (14) have reported, on the basis of roentgenological observations, that after rapid blood infusions the diastolic size does increase temporarily with a concomitant increase in the venous pressure. Beck and Holman (3) and Gregg and Wiggers (10) reported increases in diastolic size after slower blood transfusion. According to the results presented here, if the assumption can be made that diastolic size increased with increased right auricular pressure in the blood infusion experiments, then the direct relationship of cardiac output to diastolic volume cannot be accepted in these experimental conditions.

Despite the evidence presented above, the objection could still be raised that in all of these experimental situations the relations were obscured by the presence of hypervolemia. To test the critical importance of this factor, experiments have been designed to determine the effect of variations in hematocrit on cardiac output under conditions of normovolemia (22). It appears that when the blood volume is increased by blood infusion, the RAP

rises but the cardiac output does not. When the blood volume is kept constant, a decreased hematocrit and increased cardiac output occur together more consistently than do an increased cardiac output and RAP. Again when the blood volume is increased by plasma and the cardiac output and RAP have risen as the hematocrit decreased, infusion of packed erythrocytes produces further increases in RAP, but the cardiac output and hematocrit return to normal. There appears to be a relation between increased blood volume and RAP, but the cardiac output may be increased or not, depending on the hematocrit. This suggests that the RAP is relatively unimportant compared to changes in the hematocrit (and hence in the arterial oxygen content) in the regulation of cardiac output.

In contrast to the acacia experiments (8) in which the total peripheral resistance invariably decreased, in most of the blood infusion experiments the small increase over control values was not significant.

In the blood infusion experiments the hematocrit value did show some increase. Increases in blood viscosity (hematocrit increase) cause decreases in blood flow (20, 26, 15). Seligman *et al.* (20) found that as the hematocrit was increased from 40 to 75% the cardiac output decreased; they attributed this decrease to the increase in the apparent viscosity of the blood; no cardiac outputs were recorded at hematocrits lower than 32%. Whittaker and Winton (26) observed that the blood flow in the perfused hind limb of the dog was much more affected by hematocrit changes in the above-normal range than it was by decreasing the hematocrit below 40%. In the plasma and gum acacia infusions the hematocrit usually decreased from normal to below 20%.

Although change in viscosity may be a factor in the effects of these infusions, it does not appear to be the main one. This conclusion is substantiated by the observation in dogs with normal blood volume that with carboxyhemoglobinemia and methemoglobinemia the cardiac output becomes a function of the oxygen-carrying capacity of the blood. An increase in cardiac output was observed only after approximately 30% of the circulating hemoglobin had been rendered incapable of carrying oxygen (2, 17, 23). Because in those experiments the viscosity and the volume of the blood were not altered and only the oxygen-carrying capacity was decreased, the importance of arterial oxygen becomes very evident.

Under certain conditions which can be imposed on the intact dog, the generalization that there is always a direct relation between cardiac output and RAP does not hold. The unqualified application of this concept to the intact animal and to man (13, 18, 21) is, therefore, questionable.

Acknowledgment

The authors wish to thank Professors J. A. F. Stevenson, O. G. Edholm, and G. W. Manning for their interest and encouragement in this work. They are also very grateful for the technical assistance given by Messrs. G. C. Stewart, K. Jennings, and the late R. Abel.

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NEUTRAL FAT ABSORPTION IN THE RAT

THE ALLEGED EFFECT OF CHOLINE AND THE CHANGES IN THE INTESTINAL LYMPH[1]

By Ronald R. Tasker

Abstract

The intestinal absorption of olive oil was studied in the rat by cannulating the intestinal lymph trunk. After a 1 ml. test meal, the rate of flow of lymph as well as the concentration of lymph phospholipids and total lipids rose sharply, returning to fasting levels in about 20 hr. The total lipids rose and fell more sharply than the phospholipids; the concentration of lymphatic proteins remained unchanged. Roughly two-thirds of the fat meal was recovered from the lymph. The addition of 10 mgm. choline chloride to the test meal had no appreciable effect on any of the factors studied and in particular did not alter the rate of lymphatic absorption of olive oil from the intestine significantly.

Introduction

Mottram, Cramer, and Drew, in 1922 (10), reported that B vitamins altered the histological picture of fat absorption in the intestinal villi from one of scattered fat droplets to one of "absorption in streams." Cramer and Ludford (6), using "marmite", confirmed the earlier work and claimed that this vitamin supplement produced an over-all increase in the amount of stainable fat in the intestinal mucosa. Evidence was then advanced that the choline phosphatide, lecithin, increased the rate of vitamin A absorption (1) although Volk and Popper (15) subsequently showed that choline alone did not. Frazer, in 1946 (7), using choline instead of marmite described histological findings similar to those of Cramer and Ludford which suggested to him that the lipotrope increased the rate of fat absorption in the rat—findings that we in this laboratory were unable to confirm (13). From investigations in man Auger *et al.* (2) concluded that large doses of crude lecithin increased both the digestibility and the absorption of fat, while Tidwell (14) by counting chylomicra demonstrated what he considered a small increase in the rate of fat absorption after adding choline to the fat meal. Frazer (8), in his comments on our work (13), advanced biochemical data to support his concept. Nevertheless subsequent work by Shoshkes and co-workers (11) failed to indicate that choline or soybean phosphatide given orally or by intubation to normal or choline-deficient rats had any effect on fat absorption. The present experiment was designed to study the problem from a new approach involving the measurement of lipids in the intestinal lymph under various dietary conditions in acute experiments.

[1] *Manuscript received November 25, 1954.*

Contribution from the Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario. Supported in part by a grant from the National Research Council of Canada.

Methods

The intestinal lymph-trunks of unfasted rats of the Wistar strain (150-300 gm.) were cannulated by a method previously devised in our laboratory employing an artificial glass lymph-cistern (12). Following a 24–48 hr. postoperative fast, rats with a satisfactory flow of lymph were intubated as follows:

1. Ten females (average weight 240 gm.) were given 1 ml. olive oil plus 1 ml. 1% aqueous choline chloride.

2. Eleven females and one male (average weight 243 gm.) were given 1 ml. olive oil plus 1 ml. distilled water (five rats) or 1 ml. olive oil plus 1 ml. 0.37% aqueous sodium chloride, isotonic with the choline solution above (seven rats).

Lymph was collected during the antecibal fast and at intervals 0–2, 0–3, 3–6, 6–12, 12–24, and 24–48 hr. after the test meal. The glass cistern was rinsed after each evacuation with normal saline, while a 1/3000 concentration of Zephiran (brand of high molecular alkyldimethyl benzylammonium chloride) was maintained in the vessel to inhibit microorganisms.

Lymph samples were extracted with 20 volumes of 3 : 1 ethanol : ether, the residue (fat-free dry weight) being considered chiefly protein. The extract, after rectification with petroleum ether, was used for the determination of total lipids and phospholipids (9).

The weight of total lipids isolated from the lymph of rats fed oil and choline, over and above the fasting content, amounted to 29–113 (average 71)% of the fed rat while 38–109 (average 66)% was isolated from the controls.

Results and Discussion

1. *Choline and the Rate of Neutral Fat Absorption*

In Table I and Fig. 1 the rate of flow of total lipids in the intestinal lymph is recorded in grams per hour for both the control rats and those given choline. Because of the small series and the spread of the data the difference between the two groups is of questionable statistical significance. However it is apparent that this is at least not great. Since, during absorption, virtually all of the lipids in the lymph are absorbed fat (4), these data are a measure of the rate of lymphatic absorption of the olive oil fed. Moreover the bulk of experimental evidence indicates that this lymphatic absorption route is the chief one for neutral fat (3, 5). Therefore the effect of choline, if any, on the rate of olive oil absorption in the rat is small.

2. *Choline and Lymphatic Phospholipids*

Corresponding data for lymphatic phospholipids are shown in Table II and Fig. 2. There is no significant difference between the choline and control groups. Hence the lipotrope has no effect on this lipid fraction which is known to consist almost entirely of choline phosphatides during fat absorption (5).

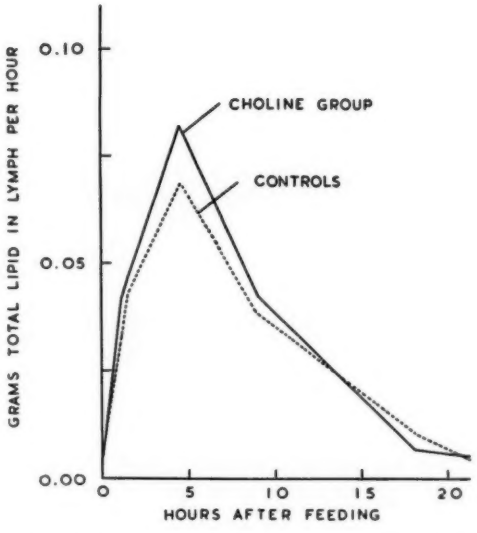

FIG. 1. The effect of choline on the rate of flow of total lipids in the intestinal lymph of the rat after a test meal of 1 ml. olive oil (gm. per hr.).

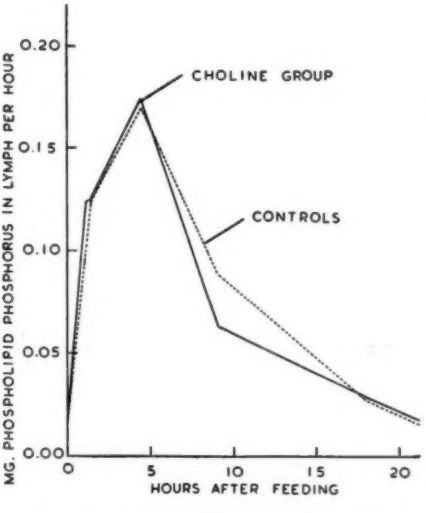

FIG. 2. The effect of choline on the rate of flow of phospholipids in the intestinal lymph of the rat after a test meal of 1 ml. olive oil (mgm. phospholipid phosphorus per hr.).

TABLE I

THE EFFECT OF CHOLINE ON THE RATE OF FLOW OF TOTAL LIPIDS IN THE INTESTINAL LYMPH OF THE RAT AFTER A TEST MEAL OF 1 ML. OLIVE OIL (GM. PER HR.)

		Hours after feeding					
	Fasting	0–2	0–3	3–6	6–12	12–24	24–48
Rats fed choline plus olive oil	.0055	.0493	.0701	.1010	.0269	.0058	.0054
	.0038	.0128	.0232	.0798	.0723	.0100	.0086
	.0100	.0596	.0660	.1600	.0610	.0072	.0014
	.0022	.0438	.0544	.1380	.0505	.0056	.0046
	.0037	.0325	.0469	.0920	.0404	.0061	
	.0054	.0525	.0545	.0221	.0369	.0136	
	.0057		.0190	.0416	.0240	.0055	
	.0059		.0629	.0533	.0284	.0081	
	.0055		.0246	.0798		.0052	
				.0509		.0021	
Means	.0053	.0418	.0469	.0819	.0426	.0069	.0050
Control rats	.0108	.0428	.0351	.0232	.0533	.0216	.0042
	.0063	.0252	.0460	.0940	.0870	.0044	.0041
	.0048	.0120	.0488	.0966	.0232	.0108	
	.0047	.0452	.0300	.0487	.0313	.0042	
	.0035	.0374	.0520	.1350	.0505	.0073	
	.0045	.0400	.0412	.0817	.0252	.0154	
	.0051		.0568	.0203	.0294	.0145	
	.0051		.0246	.0866	.0462	.0041	
	.0045		.0532	.0667	.0187	.0192	
	.0035		.0236	.0283	.0221	.0087	
	.0046		.0476	.0733		.0068	
Means	.0052	.0338	.0427	.0686	.0387	.0106	(.0042)

TABLE II

THE EFFECT OF CHOLINE ON THE RATE OF FLOW OF LYMPH PHOSPHOLIPID AFTER A TEST MEAL OF 1 ML. OLIVE OIL (MGM. PHOSPHOLIPID PHOSPHORUS PER HR.)

		Hours after feeding					
	Fasting	0–2	0–3	3–6	6–12	12–24	24–48
Rats fed choline plus olive oil	.039	.201	.234	.296	.006	.012	.018
	.010	.097	.106	.321	.090	.026	.017
	.016	.093	.135	.147	.025	.025	.017
	.010	.113	.164	.223	.087	.021	
	.017	.109	.041	.077	.119	.046	
	.012		.112	.092	.052	.014	
	.014		.081	.127	.043	.018	
	.029			.120	.079	.012	
				.165		.072	
Means	.018	.123	.125	.174	.063	.027	(.017)
Control rats	.042	.070	.097	.015	.002	.015	.016
	.023	.113	.112	.188	.163	.013	.014
	.021	.060	.094	.334	.179	.047	
	.014	.148	.072	.119	.076	.010	
	.025	.079	.144	.254	.010	.016	
	.022	.144	.143	.174	.139	.059	
	.012		.102	.082	.082	.049	
	.011		.180	.264	.095	.008	
	.022		.068	.129	.079	.045	
			.084	.086	.067	.043	
			.166	.219	.090	.017	
Means	.021	.102	.124	.170	.089	.029	(.015)

3. *Changes in the Intestinal Lymph during Olive Oil Absorption*

Table III and Fig. 3 show the alterations in the flow of lymph (ml. per hr.), lymph fat-free dry weight (gm. per hr.), lymph total lipid (gm. per hr.), and lymph phospholipid (mgm. phospholipid phosphorus per hour) and their interrelationships after a test meal of olive oil. The average results are given for 11 control rats, the findings in the group given choline being similar. There is an immediate increase in all four factors. The hourly rate of lymph flow reaches its peak within two to three hours. A parallel increase occurs in the amount of fat-free dry residue (chiefly protein) with no change in the concentration. By four to five hours the total fat (gm. per hr.) and phospholipid fractions (mgm. phospholipid phosphorus per hr.) reach their peaks.

TABLE III

THE BEHAVIOR OF INTESTINAL LYMPH AFTER A TEST MEAL OF 1 ML. OLIVE OIL IN THE RAT
RESULTS AVERAGED FOR 11 CONTROLS

		Hours after feeding					
	Fasting	0–2	0–3	3–6	6–12	12–24	24–48
Fat-free dry weight (gm. per ml. lymph volume)	0.016	0.017	0.021	0.016	0.015	0.014	(0.009)
Mgm. lymph phospholipid phosphorus divided by gm. lymph total lipid	4.4	2.9	2.3	2.5	2.6	3.1	(3.6)

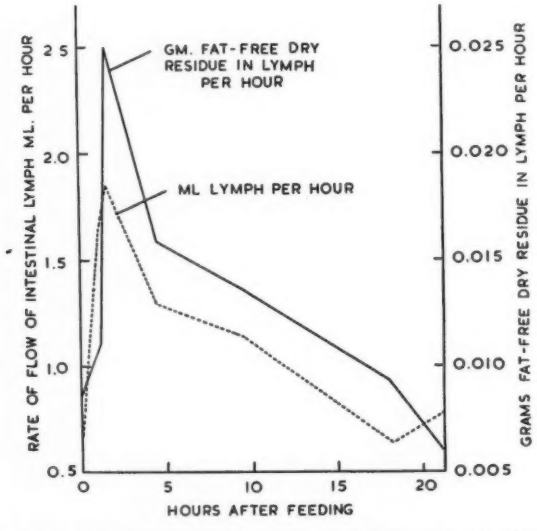

FIG. 3. The behavior of intestinal lymph after a test meal of 1 ml. olive oil in the rat—lymph volume and fat-free dry residue (ml. and gm. per hr. respectively).

These data illustrate the lymphagogue action of a fat meal and the close parallelism between the flow of lymph and of protein and between lymph total fat and phospholipid. The ratio between the former two is constant throughout, suggesting that the increased lymph flow is due, not to a change in capillary permeability, but to an increase in the rate of capillary diffusion. The ratio between phospholipid and total fat is not constant, varying between a high fasting level of 4.4 and a minimum of 2.3 at the height of absorption. This indicates that the total lipids (mostly triglyceride) rise and fall more rapidly than do the phospholipids.

Summary and Conclusions

1. Choline did not exert a pronounced effect on the rate of olive oil absorption in the intestinal lymph in the rat—the chief route of intestinal absorption for triglyceride.
2. Choline did not alter the rate of flow of phospholipid (chiefly choline phosphatide) in the intestinal lymph after a test meal of olive oil.
3. The changes in the intestinal lymph in the rat during olive oil absorption are illustrated and briefly discussed.

Measurements of quantity and composition of intestinal lymph have failed to provide any evidence that choline influenced significantly the intestinal absorption of fat in rats under the experimental conditions described. The contradictory conclusion reached by Frazer (7, 8) would appear to need further clarification in the light of these experiments and others previously reported by us (13).

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A CHEMICAL PROCEDURE FOR DETERMINATION OF THE C^{14}-DISTRIBUTION IN LABELLED XYLOSE[1]

By Stewart A. Brown

Abstract

A series of reactions reported previously for the degradation of glucose has been modified and extended to permit the determination of carbon-14 in each of the five carbons of a single 2 mM. xylose sample. Methyl xylopyranoside was oxidized with periodic acid giving C-3 as formic acid, and a dialdehyde which was converted to strontium methoxy-diglycolate. The purified salt was hydrolyzed to glyoxylic and glycolic acids. The glyoxylic acid was isolated as the 2,4-dinitrophenylhydrazone (C-1 + C-2) which was decarboxylated to give carbon dioxide from C-2. The glycolic acid was oxidized by lead tetraacetate to give C-4 as carbon dioxide and C-5 as formaldehyde. The activity in C-1 was determined by difference. The method was applied to xylose-1-C^{14}, xylose-5-C^{14}, and a biologically synthesized xylose sample with satisfactory results. This degradation procedure is theoretically applicable to other aldopentoses and aldotetroses.

Introduction

Until very recently there has been a lack of suitable procedures for complete degradation of pentoses on a millimole scale. Altermatt, Blackwood, and Neish (1) have developed a procedure involving the conversion of pentoses to lactic and acetic acids by fermentation with *Leuconostoc mesenteroides*, and degradation of each of these acids by previously described chemical methods to obtain the C^{14}-content of each of the five carbons. Although this procedure appears to yield satisfactory results, a purely chemical degradation is also desirable for use when facilities for carrying on fermentations are not readily available. The procedure recently reported from these laboratories by Boothroyd and co-workers (2), based on the oxidation of methyl glucoside with periodic acid, has suggested such a method. This series of reactions has now been modified and extended to permit the determination of the C^{14}-content of each of the five carbons of xylose, as shown in Fig. 1. A 2 mM. sample of xylose, containing less than 0.5 μc. of C^{14}, can be conveniently degraded.

The sugar is first converted to methyl xylopyranoside (I, Fig. 1) with methanolic hydrogen chloride, and the xyloside is oxidized with periodate (5). The resulting dialdehyde (II), a mixture of α- and β-anomers representing all carbons but C-3, is oxidized with bromine to methoxy-diglycolic acid which is isolated as the strontium salt (III). On hydrolysis this yields glyoxylic acid (IV) (C-1 + C-2) and glycolic acid (V) (C-4 + C-5). The glyoxylic acid is further degraded as previously described (2) and the glycolic acid is oxidized with lead tetraacetate to carbon dioxide (C-4) and formaldehyde (C-5). Carbon-3 is recovered as formic acid following oxidation of the methyl xyloside.

[1] *Manuscript received January 4, 1955.*
Contribution from the Prairie Regional Laboratory, National Research Council, Saskatoon, Sask. Issued as N.R.C. No. 3577.

The validity of the above procedure has been checked by degradation of xylose samples specifically labelled in C-1 and C-5, and of a sample of biologically synthesized xylose which had also been degraded by the fermentation procedure.

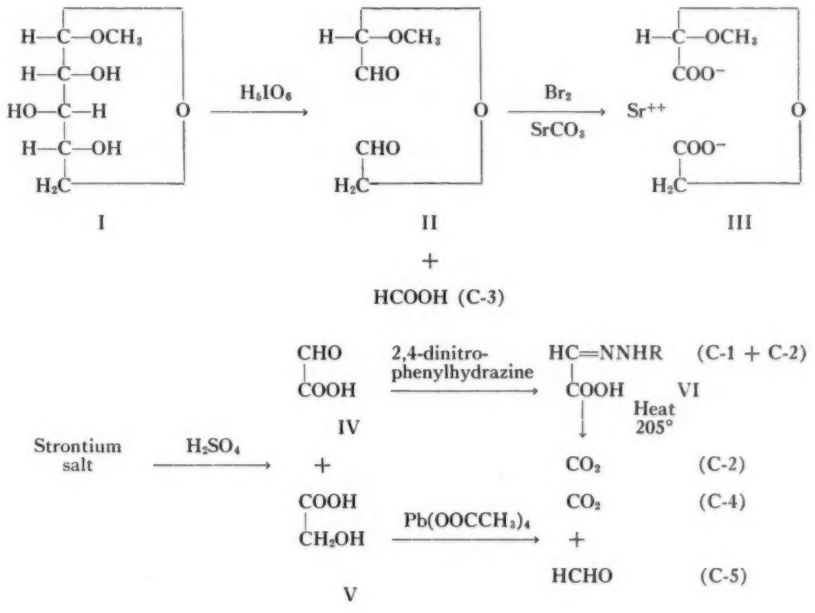

FIG. 1. Chemical degradation of methyl-D-xyloside. R in VI is the 2,4-dinitrophenyl radical. C-1, C-2, etc. refer to the position of the carbon atom in the xylose molecule.

Experimental

Methyl-D-xylopyranoside

In the earlier experiments reported here the procedure of Hudson (4) was followed in preparing and isolating the methyl xylosides. These were purified by recrystallization to remove any unreacted free xylose which, if allowed to remain, would have resulted in contamination of the formate formed from C-3 by the periodate oxidation of the xylosides. Although this procedure was satisfactory from the standpoint of purification it was more time-consuming than the analogous synthesis reported for glucose (2), because both anomers must be isolated to obtain enough material for degradation. The finding of Roseman *et al.* (7) that reducing sugars could be removed from solution by strongly basic anion exchange resin suggested the possibility of purifying the methyl xylosides without the necessity of their isolation. This proved entirely satisfactory, and the following method has been adopted.

Three hundred milligrams (2 mM.) of dry α-D-xylose was dissolved in 3 ml. of dry methanol containing 1% hydrogen chloride and the solution was refluxed for six hours (4). It was then diluted with three parts of water and passed through a 1.5 × 10 cm. column of Amberlite IR-400 anion exchange resin at a rate not exceeding 5 ml. per min. This treatment removed the hydrochloric acid as well as most of the free xylose in the solution. The column was washed with 40 ml. of carbon dioxide-free water, and the eluate and washings containing the methyl xylosides were concentrated *in vacuo* to a heavy sirup, which was taken up in 4.5 ml. of water.

Oxidation of the Methyl Xylosides and Determination of C-3

To the solution of methyl xylosides was added 10.5 ml. of 0.4 *M* periodic acid (final concentration *ca.* 0.27 *M*) and the oxidation was allowed to proceed in the dark for nine hours, or overnight. The reaction solution was passed through a column of Amberlite IR-4B resin to remove iodic and periodic acids, and the formate representing C-3 was eluted with 0.2 *N* barium hydroxide and oxidized to carbon dioxide (2).

Strontium Methoxy-diglycolate

The first eluate and washings from the resin column, containing methoxy-diglycolic aldehyde, amounted to 65 to 75 ml. This solution was treated with 0.8 ml. of bromine in the presence of 4 gm. of strontium carbonate for 30 min., with continuous mechanical stirring. The oxidation mixture was purified as described previously (2) and the solution was concentrated to between 1 and 2 ml. The addition of a large excess of absolute ethanol with vigorous stirring resulted in the immediate separation of a white precipitate of crude strontium methoxy-diglycolate trihydrate. Usually this was in the form of a semicrystalline mass which crystallized completely within a minute or two. The yield was 300 mgm., 50% of theoretical based on xylose. For recrystallization, the product was taken up in 5 ml. of hot 30% ethanol and filtered free of a small amount of flocculent material. The filtrate was reheated, and 6 to 7 ml. of hot absolute ethanol was added slowly. The solution was allowed to cool, with frequent stirring until crystallization was initiated; this overcame a tendency sometimes observed for the strontium salt to separate as a sirup. The recrystallized material weighed 255 mgm.

Glyoxylic Acid 2,4-Dinitrophenylhydrazone and Glycolic Acid

The purified strontium salt (220 mgm., 0.725 mM.) was hydrolyzed with 4 ml. of *N* sulphuric acid for 1.5 hr. on the steam bath. The precipitate of strontium sulphate was filtered off and washed, and the filtrate and washings were diluted to about 40 ml. To this was added a solution prepared by dissolving 160 mgm. of 2,4-dinitrophenylhydrazine in 1 ml. of concentrated sulphuric acid and diluting with an equal volume of water. The mixture was refrigerated for an hour and filtered. The residue of glyoxylic acid 2,4-dinitrophenylhydrazone (VI) and the filtrate containing glycolic acid were retained and treated as described below.

Determination of C^{14} in C-1 and C-2

The glyoxylic acid 2,4-dinitrophenylhydrazone was purified and degraded as previously reported (2). The purified product weighed 161 mgm., 88% of theoretical based on the strontium salt.

Determination of C^{14} in C-4 and C-5

The glycolic acid solution was passed through a column of IR-120-H cation exchange resin, which was washed with water until the pH of the effluent was 5 to 6. The color remaining after this treatment disappeared after the solution had been stirred briefly with decolorizing charcoal. The sulphuric acid in the solution was neutralized by barium hydroxide followed by barium carbonate and the mixture was filtered. The filtrate and washings were concentrated to about 20 ml. *in vacuo*, and passed through a small (1 × 7 cm.) IR-120-H resin column to remove the barium ions. The eluate and washings were then concentrated to about 6 ml. and 24 ml. of glacial acetic acid was added. This solution was added all at once to 30 ml. of glacial acetic acid containing 600 mgm. of lead tetraacetate and 600 mgm. of potassium acetate (6), in a 125 ml. flask equipped with two side arms. The flask was closed and, after mixing, the oxidation was allowed to proceed at room temperature for 5.5 hr. One side arm of the flask was then connected to a source of carbon dioxide-free nitrogen, and the other through a trap immersed in an ice–salt mixture to an absorption tower containing sodium hydroxide solution. The flask was shaken vigorously for one-half hour on a mechanical shaker[2] while being swept vigorously with nitrogen. Any acetic acid carried over was condensed in the trap and never reached the absorption tower. The trapped carbon dioxide was recovered for counting as barium carbonate. (Yield: 120 mgm., 84% of theoretical based on the strontium salt.) The above procedure resulted in quantitative yields of carbon dioxide from known samples of glycolic acid, and the degradation of xylose-5-C^{14} showed that less than 1% of the formaldehyde produced in the oxidation was further oxidized to carbon dioxide.

The lead was removed from the residual solution by the addition of 350 mgm. of oxalic acid dissolved in 5 ml. of glacial acetic acid, followed by filtration. The filtrate was diluted with about nine volumes of water, an excess of dimedon solution (2) was added, and concentrated sodium hydroxide solution was poured in slowly, with stirring and cooling, until at about pH 6 a milkiness developed. Crystallization of formaldimedon, which began almost at once, was completed in the refrigerator. The product was recrystallized once from 50% ethanol. (Yield: 125 mgm., 60% of theoretical based on the strontium salt.)

Measurement of Radioactivity

All determinations of carbon-14 were carried out with the same apparatus and techniques as described in a previous paper (2).

[2] *It is imperative that the flask be* shaken. *Even vigorous* stirring *is ineffective in releasing carbon dioxide from the solution.*

Results and Discussion

Table I shows the results of C^{14} analyses obtained from the degradation of xylose samples labelled specifically at C-1 and C-5, and of a sample of xylose[3] isolated from wheat hemicellulose. In all three cases the recovery of C^{14} based on specific activities was in excess of 95%, and in the two specifically labelled samples the contamination appearing in originally unlabelled carbons did not exceed about 1% of that in the labelled carbon. This contamination is negligible, being within the counting error. The value of the specific activity of C-1 determined after degradation of xylose-1-C^{14} is a conservative one, as in other runs it approached more closely that of the methyl xyloside.

TABLE I

DETERMINATION OF C^{14} DISTRIBUTION IN XYLOSE

Compound	D-Xylose-1-C^{14}		D-Xylose-5-C^{14}		D-Xylose-C^{14} from wheat hemicellulose	
	μc./mM.	% of total	μc./mM.	% of total	μc./mM.	% of total
α-D-Xylose	—	—	—	—	0.340	100
Methyl-D-xyloside	0.106	100	—	—	—	—
Strontium methoxy-diglycolate	0.102	96.3	0.434	100	—	—
Glyoxylic acid 2,4-dinitrophenylhydrazone (C-1 + C-2)	0.994	93.8	0.0006	*0.1*	0.1384	40.7
C-1*	0.993	*93.7*	—	—	0.0685	*20.1*
CO_2 from:						
C-2	0.001	*1.2*	—	—	0.0699	*20.6*
C-3	0.0006	*0.6*	0.0005	*0.1*	0.0707	*20.8*
C-4	0.0003	*0.3*	0.004	*0.9*	0.0705	*20.7*
C-5	0.001	*0.1*	0.423	*97.5*	0.0648	*19.1*
Total†		95.9		98.6		101.3

* *Obtained by difference.*
† *Of italicized numbers.*

The results for the biologically synthesized sample were compared with those obtained from a degradation of another aliquot of the same sample by Dr. H. A. Altermatt, using the *Leuconostoc* fermentation method (1). The comparative values did not differ for any one of the five carbons by more than 5.5%, and the average deviation was less than 3%. The validity of the present procedure thus appears established, in so far as this is possible without having available samples of xylose labelled specifically in each of the five possible positions.

Appreciable blanks exist for carbons 2, 3, and 4, which are isolated as barium carbonate. These evidently result from the presence of small amounts

[3] *This sample was provided through the courtesy of Dr. Gleb Krotkov of Queen's University, Kingston, Ont.*

of carbon dioxide and carbonates in the air and in the reagents, and in the case of C-4 there is also probably a slight oxidation of the acetic acid used as solvent in the lead tetraacetate oxidation. For accurate estimations of specific activities, occasional blank values should be determined; these should not change significantly as long as the experimental conditions are not appreciably altered. We obtained the C-3 blank by passing a solution of 704 mgm. of iodic acid (HIO_3) in 15 ml. of water plus 0.5 ml. of 0.4 *M* periodic acid through an IR-4B resin column, eluting with barium hydroxide solution and oxidizing the eluate with mercuric oxide under the usual conditions.

The technique described above is theoretically applicable to any aldose the methyl glycoside of which can be converted to strontium methoxydiglycolate. This includes the aldopentoses, and erythrose (3); no report of the preparation of this strontium salt from threose has been found. A methyl tetroside on periodate oxidation would, of course, yield no formic acid. About four days are required, after a little experience, to prepare all five carbons for counting, and two or more samples could be run concurrently.

There is an alternative method[4] for separating the hydrolysis products of the strontium salt which is theoretically simpler than the one described above and which allows the direct measurement of the C^{14} content of all five carbons. This involves treatment of the mixture of glyoxylic and glycolic acids with periodic acid, which should oxidize the former quantitatively while leaving the latter unattacked. The C-1 formate is then oxidized with mercuric ion and the filtrate from this treatment is worked up for degradation of the glycolic acid. In practice, however, when a sample of xylose-1-C^{14} was degraded C-4 was contaminated with about 6% of the specific activity of C-1, apparently because of incomplete oxidation of the C-1 formate, or possibly of the glyoxylic acid. Several variations of the technique failed to overcome the difficulty, and the method was abandoned in favor of separation of the two-carbon fragments.

Acknowledgments

The author wishes to thank Dr. A. S. Perlin of these laboratories for valuable information about experiments with lead tetraacetate oxidations carried out with Dr. C. T. Bishop in Ottawa. He is also grateful to Dr. A. C. Neish for several helpful discussions of the problem and for providing the specifically labelled xylose, and to Messrs. F. M. Clare and John Dyck for technical assistance.

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[4] *This was suggested by Dr. A. S. Perlin in a private communication.*

THE EFFECT OF CORTICOTROPIN AND CORTICOSTERONE ON THE PRODUCTION OF HEMOLYTIC ANTIBODIES IN THE MOUSE[1]

By Shirley E. Newsom and Marvin Darrach

Abstract

It has been demonstrated by quantitative immunochemical methods that corticotropin, when administered at sufficiently high levels, will suppress the formation of circulating hemolytic antibodies in the mouse; this apparent block on the antibody producing mechanism can be overcome by increasing the dose of antigen. Furthermore, it has been shown that corticosterone, which appears in greater amounts in the blood of mice after treatment with corticotropin, will also suppress the normal rise of circulating immune hemolysins in the mouse.

Introduction

Quantitative immunochemical data resulting from recent investigations have shown that cortisone acetate inhibits the formation of circulating hemolytic antibodies in the mouse and that the extent of the observed inhibition depends upon the dose of both antigen and hormone (5).

Similar quantitative methods have now been used to determine whether the adrenal stimulating hormone, corticotropin, can exert a similar antibody suppressing effect. Other investigators have demonstrated by immunochemical techniques that this hormone inhibits, to some extent, the normal rise of circulating antibodies to egg albumin (3) and pneumococcal vaccine (1) in the rabbit.

It has been demonstrated by Southcott *et al.* (6) in these laboratories that corticotropin markedly increases the blood level of corticosterone in the mouse and, further, that corticosterone is by far the most abundant if not the only unconjugated circulating corticosteroid in mouse blood. In view of these findings, it became of additional interest to study the effect of this steroid on the formation of circulating hemolytic antibodies.

Experimental

The preparation of antigen and immune sera and the quantitative comparison of hemolysin levels have been described in detail (5, 2, 4). Unless otherwise indicated, antigen was administered by the intraperitoneal route at the standard dose of a single injection of 0.1 ml. of a 1% suspension of washed sheep erythrocytes; after a six-day immunization period, all mice were bled by cardiac puncture and the sera were pooled from appropriate groups of 10 or 20 female Swiss mice which weighed 18–20 gm. at the start of each experiment.

[1] *Manuscript received January 17, 1955.*

Contribution from the Department of Biochemistry, Faculty of Medicine, University of British Columbia, Vancouver, B.C.

Effect of Different Levels of Corticotropin on Production of Circulating Hemolytic Antibodies in the Mouse

Corticotropin* treatment consisted of two subcutaneous injections daily, starting one day prior to the administration of the sheep cell antigen and continuing throughout the entire six-day immunization period. Control studies demonstrated that the corticotropin vehicle,† when administered alone at the highest levels used in the following experiments, had no significant influence on the production of circulating hemolytic antibodies.

Preliminary trials on groups of 10 mice showed that treatment with less than 1.0 I.U. per day of corticotropin did not suppress the normal rise of immune hemolysins in response to the standard dose of antigen. Although lower than normal levels of circulating hemolysins resulted from a dose of 1.0 I.U. of corticotropin per day, the apparent drop was not far beyond the variation frequently encountered in control levels and was, therefore, of doubtful significance. Experiments with larger doses of hormone were then conducted in order to determine whether increasing amounts of corticotropin would be accompanied by decreasing levels of antibody, as was found in previous experiments with cortisone acetate, and whether the suppression could be brought near to completion with a sufficiently large dose of corticotropin.

TABLE I

EFFECT OF TWO SUBCUTANEOUS INJECTIONS OF CORTICOTROPIN PER DAY ON CIRCULATING HEMOLYTIC ANTIBODY LEVELS IN FEMALE SWISS MICE

	Control animals		Corticotropin treated animals				
Experiment	No. of mice in group	Hemolytic antibody level of pooled sera, O.D./min./ml.	Dose, I.U./day	Lot No.	No. of mice in group	Hemolytic antibody level of pooled sera, O.D./min./ml.	Per cent suppression from normal levels
1	20	.080	1.0	53004	8	.058	27.5
			2.0		10	.041	48.8
2	20	.147	2.0	53009	10	.058	60.6
			4.0		10	.002	98.6
3	20	.280	4.0	54005	9	.068	75.7
			4.0		9	.033	88.2
4	20	.232	4.0	54005	9	.018	92.3
			4.0		8	.015	93.5
			6.0		8	.007	97.0
5	20	.094	6.0	54015	10	.005	94.7
			6.0		10	.011	88.3

* *DURACTON (Corticotropin—carboxy methyl cellulose) derived from pork anterior pituitary, supplied by Nordic Biochemicals, Ltd., Montreal, Canada.*

† *DURACTON CMC, supplied by Nordic Biochemicals.*

In each of five separate experiments, involving different batches of antigen, 20 mice served as controls for test groups consisting of 10 mice. In the first study, two test groups received 1.0 and 2.0 I.U. of corticotropin daily; in the second, 2.0 and 4.0 I.U.; in the third, 4.0 and 4.0 I.U.; in the fourth, 4.0, 4.0, and 6.0 I.U.; and in the fifth, 6.0 and 6.0 I.U. per day. The results are given in Table I. The level of circulating antibody was consistently low in those animals receiving the 6.0 I.U. dose, and a marked decrease of hemolysins also resulted from 4.0 I.U., while 2.0 I.U. gave only partial inhibition. Fig. 1 shows the per cent suppression of hemolytic antibody accompanying the various doses of corticotropin when compared with the corresponding control antisera. These data demonstrate clearly that the amount of circulating hemolysins responding to the standard dose of antigen decreases with increasing doses of corticotropin.

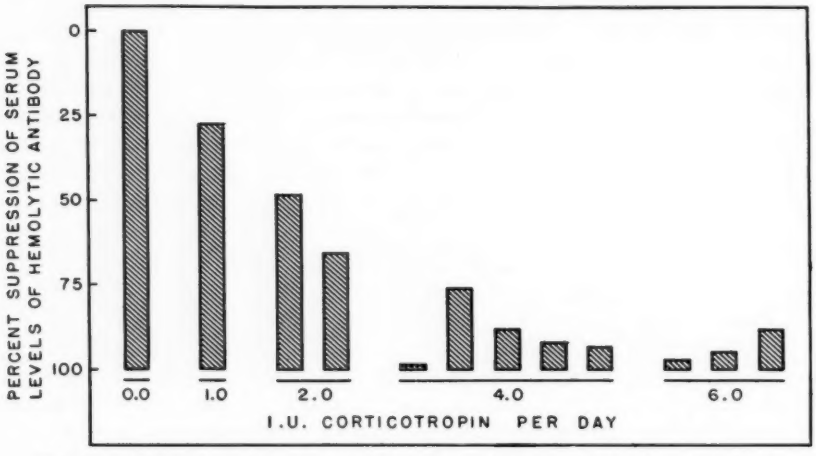


FIG. 1. Effect of increasing amounts of corticotropin on the production of circulating hemolytic antibodies in the mouse.

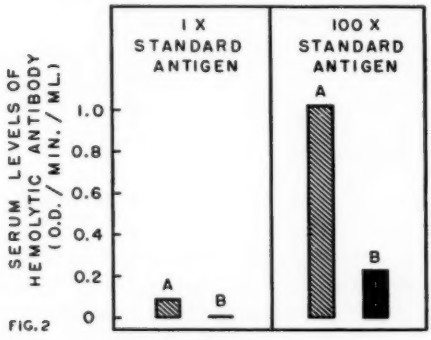
Effect of an Increased Level of Antigen on the Production of Circulating Hemolytic Antibodies in the Corticotropin Treated Mouse

In a previous study (5) it was shown that the dose of cortisone acetate which almost completely suppresses circulating hemolytic antibody levels in mice receiving the standard amount of antigen does not prevent the appearance of considerable quantities of hemolysins when the dose of anitgen is substantially increased. Therefore, a similar experiment was conducted with corticotropin to determine whether a high dose of antigen could overcome its apparent block on the production of circulating hemolytic antibody.

Four groups of 10 female mice were injected with a total dose of 6.0 I.U. of corticotropin daily over a seven-day period. On the second day of the experiment two of these groups were given the standard dose of sheep cell antigen and the remaining two were given 100 times the standard dose. Four

additional groups of 10 mice served as controls and received no hormone; two of these groups received the standard dose of antigen and two the larger dose. After a six-day immunization period the mice were bled and the sera examined for hemolytic activity.

The results are illustrated in Fig. 2 and show, as before, that daily treatment with a total of 6.0 I.U. of corticotropin will almost completely suppress the appearance of circulating hemolysins in mice on the standard dose of antigen. However, in animals receiving the same dose of corticotropin but 100 times the standard antigen dose, there are substantial amounts of circulating hemolysins, although the levels are lower than those of the corresponding control animals receiving no hormone. All mice given corticotropin in this experiment showed a marked atrophy of the thymus and spleen.



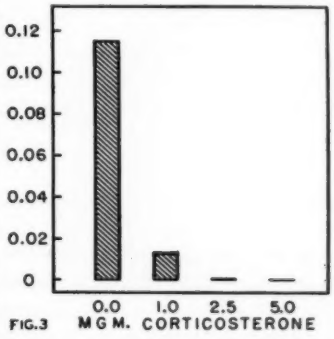


FIG. 2. Effect of an increased dose of antigen on circulating hemolytic antibody levels in mice receiving a total of 6 I.U. of corticotropin per day. A—groups receiving antigen only. B—groups receiving antigen and corticotropin.

FIG. 3. Effect of increasing amounts of corticosterone on the production of circulating hemolytic antibodies in the mouse.

Effect of Different Levels of Corticosterone on the Production of Circulating Hemolytic Antibodies in the Mouse

After it had been established that corticotropin inhibits the production of circulating hemolytic antibodies in the mouse and that a similar dose of corticotropin enhances the plasma levels of corticosterone, it became of interest to determine the effect of corticosterone on the production of immune hemolysins in the mouse.

Corticosterone* was administered as a fine suspension in saline and Twee n20† prepared in the following manner. To a solution of 100 mgm. of corticosterone in 6.5 ml. of acetone, 1.4 ml. of Tween 20 was added; the acetone was removed by evaporation at 45° C. under a stream of nitrogen and, to the clear solution, physiological saline was added slowly, with stirring, until the resulting fine suspension of corticosterone reached a volume of 4.0 ml., and a concentration of 25 mgm./ml. The indicated doses of this suspension were given to the

* *Merck and Company, Inc., Rahway, New Jersey.*
† *Atlas Powder Company, Wilmington, Del.*

mice in a single subcutaneous injection one day before the administration of antigen. Control studies showed that Tween 20 reagent blanks, prepared as above, but without corticosterone, had no significant effect on the formation of circulating hemolysins when administered at the highest levels used in the following experiments.

Preliminary experiments indicated that a dose of 5.0 mgm. of corticosterone given to mice one day before the administration of the standard amount of sheep cell antigen inhibited almost completely the appearance of circulating hemolytic antibodies. The data are given in Table II.

TABLE II

EFFECT OF A SINGLE 5.0 MGM. SUBCUTANEOUS INJECTION OF CORTICOSTERONE ON CIRCULATING HEMOLYTIC ANTIBODY LEVELS IN FEMALE SWISS MICE

Control animals		Corticosterone treated animals	
No. of mice in group	Hemolytic antibody level of pooled sera, O.D./min./ml.	No. of mice in group	Hemolytic antibody level of pooled sera, O.D./min./ml.
20	.232	10	.0012

Further studies were carried out to confirm these results and to determine the dose of corticosterone at which partial inhibition of hemolysin production occurred. Fifty female mice were divided into groups of 10. Three groups of animals received 1.0, 2.5, and 5.0 mgm. of corticosterone respectively, while two groups served as controls. After all the mice had been immunized and bled, the sera were examined quantitatively for hemolysins. The results are recorded in Fig. 3 and show that 5.0 mgm. of corticosterone cause complete suppression, 2.5 mgm. almost complete suppression, and 1.0 mgm. partial but significant inhibition of antibody formation.

Discussion

These experiments have shown that corticotropin, when administered at sufficiently high levels, can suppress the rise of hemolytic antibodies in the mouse. Thus, at the standard antigen dose of 0.1 ml. of a 1% suspension of sheep erythrocytes, the level of hemolytic antibody decreases with increasing amounts of hormone until an end point is reached where almost complete antibody suppression occurs. This takes place with a dose of corticotropin in the range of 4–6 I.U. per mouse per day. Such amounts of corticotropin induce a characteristic and marked atrophy of the spleen and thymus. However, despite these conditions, comparatively high levels of circulating hemolytic antibody appear if the dose of antigen is substantially increased. The results of these experiments and the data previously reported after a similar study with cortisone acetate (5) suggest that the adrenal secretion, although able to suppress hemolytic antibody formation, cannot maintain an

effective block when the quantity of antigen surpasses a certain limit. Thus, in spite of the atrophy of the lymphatic tissue accompanying the large doses of cortisone acetate and corticotropin used, it seems apparent that the over-all biochemical mechanisms involved in the production of circulating immune hemolysins are able to function providing sufficient antigen is present.

The mechanism of action of corticotropin in suppressing circulating hemolytic antibody formation is probably due to its stimulation of the mouse adrenal to release corticosterone. Evidence supporting this view is based on the fact that corticosterone, shown in this study to have hemolytic antibody suppressing activity, is found in increased amounts in the plasma of mice after treatment with the dose of corticotropin effecting almost complete inhibition of hemolysins to the standard dose of antigen. Although cortisone acetate had approximately the same activity (5) as corticosterone in suppressing the formation of circulating hemolytic antibody, neither cortisone nor any of the other known 17-hydroxy corticosteroids could be detected in mouse blood either before or after the administration of corticotropin. Further studies on the significance of these findings are in progress.

Acknowledgments

This study was supported by a grant-in-aid from the Division of Medical Research of the National Research Council of Canada. The corticotropin was kindly donated by Dr. K. Antoft, Nordic Biochemicals Ltd., Montreal, Canada, and the corticosterone by Dr. Augustus Gibson, Merck and Company Inc., Rahway, New Jersey. The authors also acknowledge the technical assistance given by Miss Vera Stanley and Miss Sharon Pitts to some parts of the project.

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THE INFLUENCE OF THE THYROID GLAND ON THE ISLETS OF LANGERHANS AND THE PANCREAS[1]

By B. Kinash and R. E. Haist

Abstract

When sufficiently large amounts of desiccated thyroid gland were administered to intact or hypophysectomized rats there was an increase in the weight of the pancreas, weight of the islets of Langerhans, and islet weight per unit of body weight. In intact rats the concentration of islet tissue in the pancreas was not significantly altered, but in hypophysectomized animals the concentration of islet tissue in the pancreas was reduced because of the large increase in pancreas weight. The great reduction in pancreas weight occasioned by hypophysectomy was to a large extent prevented by the administration of desiccated thyroid gland.

A number of reports have indicated that the thyroid gland may influence the growth and function of the islets of Langerhans. It has been said that removal of the thyroid gland in the rat caused a reduction in the volume and number of the islets of Langerhans (7). Administration of thyroid materials or hyperthyroidism, on the other hand, has been stated to cause an increase in the size or number of the islets in a variety of species (1, 3, 4, 7, 13). One report indicated that acute hyperthyroidism in guinea-pigs led to necrosis of islet tissue, but when thyroid extract was given over a long period of time (175–193 days) hyperplasia of the islets occurred (11).

Injections of thyroxine in intact animals in doses greater than 1 mgm. were found to cause hyperplasia and hypertrophy of the islets, but thyroid administration in partially depancreatized dogs led to diabetes (12). Degranulation and hydropic degeneration in the islets of Langerhans in dogs with thyroid diabetes have been noted (2, 8). This was in contrast with the effect of prolonged thyroid administration (60 days) in the rat, which caused a reduction in the incidence of diabetes resulting from partial removal of the pancreas and which also increased the resistance to administered alloxan (9). Histological examination seemed to show a new formation of islets or enlargement of existing islets in the remnants, and this was thought to be a factor of importance in the improvement (9).

There are species differences in the effects of thyroid administration. Also few quantitative data are available concerning the effects of thyroid administration especially in the absence of the hypophysis. It was considered desirable, therefore, to obtain quantitative data concerning the islet changes with thyroid administration in normal and hypophysectomized rats.

Materials and Methods

Male and female rats of the Wistar and Sprague-Dawley strains were used in these experiments. Desiccated thyroid gland was added to the ground diet (Master Feeds Fox Breeder Ration) and mixed thoroughly in a ball mill.

[1] *Manuscript received November 18, 1954.*
Contribution from the Department of Physiology, University of Toronto, Toronto, Ontario.

The test rats received the food containing the desiccated thyroid gland. The control rats received the same ration minus the thyroid material. The concentration of desiccated thyroid gland in the diet was usually 0.25%. In one experiment it was 0.025%. In the experiments with intact rats, both test and control animals were fed ad libitum. In tests with hypophysectomized rats the control animals were paired-fed with the animals receiving the thyroid material. Test and control rats were sacrificed at the same times from seven days to over two months. Islet weights were estimated by a slight modification of the method of Haist and Pugh (5). The staining with this procedure was found to be unsatisfactory when the period of treatment was under 30 days, this time varying somewhat with the thyroid dosage.

Experimental Results

The Effect of the Administration of Desiccated Thyroid to Intact Rats

With daily doses of desiccated thyroid gland greater than 0.25 gm. per 100 gm. of diet, the loss in body weight was great and many animals died. It was found that a consistent effect on the islets was not obtained until after

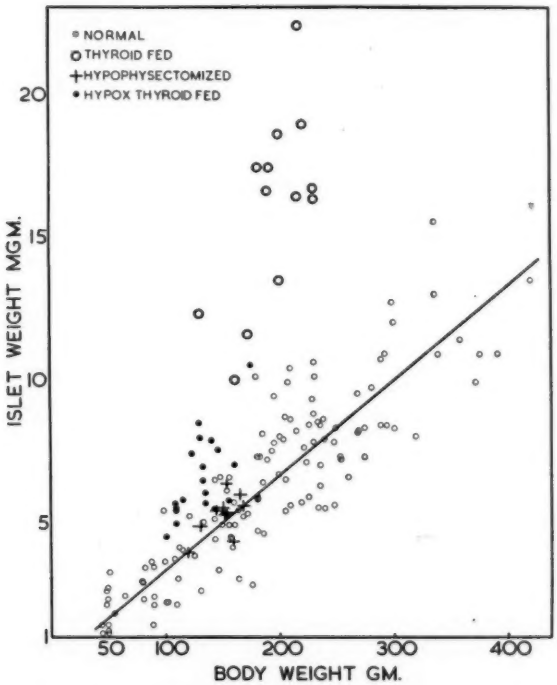

FIG. 1. The effect of thyroid administration on the islet weights. Small circles = intact controls; large circles = intact fed thyroid; crosses = hypophysectomized; solid dots = hypophysectomized, fed thyroid.

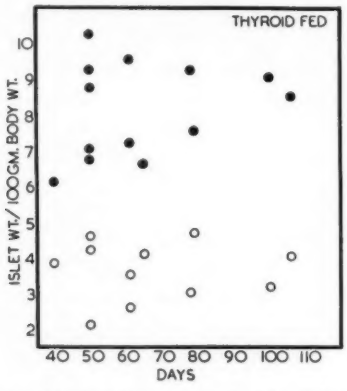

FIG. 2. The effect of thyroid administration on the islet weight per unit of body weight Circles = intact controls; solid dots = intact fed thyroid.

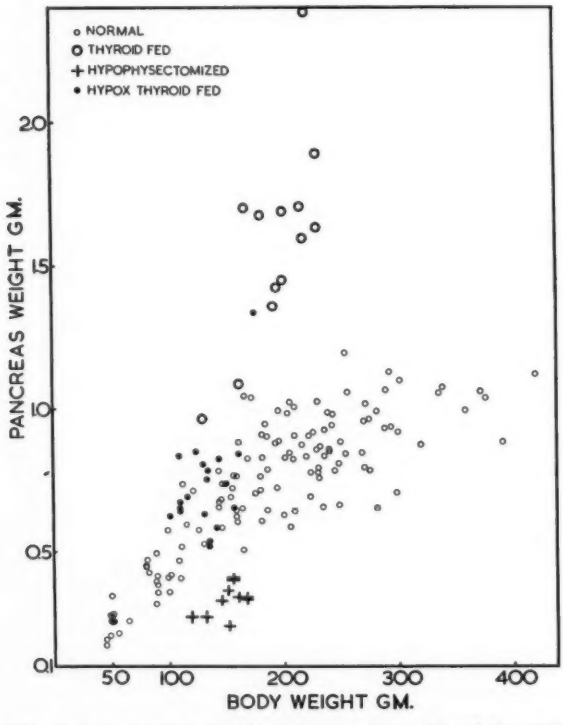

FIG. 3. The effect of thyroid administration on the weight of the pancreas. Small circles = intact controls; large circles = intact fed thyroid; crosses = hypophysectomized; solid dots = hypophysectomized, fed thyroid.

40 days of administration of the thyroid material. Results obtained when 0.25 gm. desiccated thyroid per 100 gm. of diet was given for 40 days or longer are shown in Figs. 1 to 3 and are summarized in Table I. The pancreas weight increased with the thyroid administration ($p < .001$), as did also the weight of the islets of Langerhans ($p < .001$). The islet to pancreas ratio was not significantly altered but the islet weight per 100 gm. body weight was significantly elevated ($p < .001$). This latter effect was enhanced by the depressing effect of the thyroid administration on body growth ($p < .01$). The results obtained when 0.025 gm. desiccated thyroid per 100 gm. of diet was given are shown also in Table I. No significant changes in islet weight, pancreas weight, or body weight were observed when this dosage was used.

TABLE I

THE EFFECT OF THYROID FEEDING IN INTACT AND HYPOPHYSECTOMIZED RATS

Hypox = hypophysectomized. Means are followed by standard deviations.

Group	No. of rats	Mean body weight		Mean pancreas weight, gm.	Mean islet weight, mgm.	Mean islet × 100 / pancreas wt.	Mean islet weight per 100 gm. body wt., mgm.
		Initial, gm.	Final, gm.				
1. Thyroid (low dose) (40 days)	8	161	256 ± 22	1.053 ± 0.011	10.1 ± 2.2	0.95 ± 0.14	3.9 ± 0.7
2. Control (40 days)	9	160	284 ± 46	0.944 ± 0.011	10.6 ± 2.2	1.13 ± 0.24	3.8 ± 0.8
3. Thyroid (high dose) (40 days)	13	152	196 ± 29	1.583 ± 0.358	16.0 ± 3.4	1.03 ± 0.23	8.2 ± 1.3
4. Control (40 days)	11	153	248 ± 44	0.809 ± 0.107	9.1 ± 2.8	1.12 ± 0.29	3.7 ± 0.8
5. Hypox	10	147	150 ± 5	0.333 ± 0.053	5.3 ± 0.7	1.60 ± 0.26	3.5 ± 0.4
6. Hypox + thyroid	19	149	132 ± 20	0.736 ± 0.177	6.5 ± 1.5	0.90 ± 0.19	5.0 ± 0.8
7. Controls	6	139	201 ± 16	0.817 ± 0.052	8.7 ± 0.8	1.07 ± 0.07	4.4 ± 0.4
t between 1 and 2			1.66	1.80	0.17	0.33	0.33
t between 3 and 4			3.48**	6.89***	5.37***	0.85	9.81***
t between 5 and 6			2.52†	6.99***	2.51†	8.33***	5.20***
t between 6 and 7			7.76***	1.09	3.37**	2.10*	1.67

*** $p < .001$, ** $p < .01$, † $p < .02$, * $p < .05$.

The Effect of the Administration of Desiccated Thyroid Gland to Hypophysectomized Rats

Hypophysectomized Sprague-Dawley rats were given a diet containing 0.25 gm. desiccated thyroid gland per 100 gm. of food for longer than 40 days. Control animals receiving the same caloric intake and controls fed ad libitum were used. The results are presented in Figs. 1 and 2 and are summarized in Table I. From this it will be seen that the thyroid administration in

hypophysectomized rats occasioned an increase in the islet weight ($p < .02$), pancreas weight ($p < .001$), and islet weight per 100 gm. body weight ($p < .001$). The islet to pancreas ratio was reduced by the thyroid administration ($p < .001$) because of the large increase in pancreas weight.

Discussion and Conclusions

The results of these investigations indicate that in certain strains of rat the administration of sufficiently large doses of desiccated thyroid gland for greater than 40 days enhanced the growth of the islets in the presence or absence of the pituitary gland. This stimulation of islet growth was out of proportion to changes in body weight. In the intact rat, the islet to pancreas ratio was not increased by the thyroid administration and in the hypophysectomized rat it was actually reduced by the thyroid feeding because of the large increase in pancreas weight. These observations support the conclusion of Houssay and others mentioned previously that thyroid administration stimulates islet growth. The present observations show that the effect is not mediated by the pituitary gland.

The low pancreas weights which result from hypophysectomy were not observed when desiccated thyroid was administered to hypophysectomized rats. This would seem to indicate that a large part of the effect of hypophysectomy on the external-secreting portion of the pancreas is associated with altered thyroid function, though it is unlikely that this is the sole factor involved. As noted previously by others (6, 10), thyroid administration in the intact rat led to a marked increase in pancreas weight above that of controls.

We wish to thank Professor Charles H. Best for his interest in this work and to acknowledge gratefully the financial assistance of the National Research Council of Canada.

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THE PYRUVIC PHOSPHOFERASE OF ERYTHROCYTES

II. ACTIVITY IN ANEMIC AND TUMOR-BEARING ANIMALS AND IN HUMAN BLOOD DISORDERS[1]

By P. F. Solvonuk and H. B. Collier

Abstract

Pyruvic phosphoferase (PPFase) activity of the erythrocytes (RBC) was determined in rabbits and rats made anemic by acetylphenylhydrazine injection or by bleeding, in rats bearing the Walker 256 carcinoma, and in human patients suffering from anemia, including several cases of pernicious anemia. An elevation of the PPFase activity was found to accompany the appearance of new RBC in the circulation, but the enzyme level was not always related to the proportion of reticulocytes or the mean RBC volume. Administration of vitamin B_{12} to a previously untreated case of pernicious anemia resulted in a marked increase in the PPFase activity during regeneration of RBC. The highest PPFase activity was found in a patient with nutritional microcytic anemia. It is concluded that a high PPFase activity is associated with immature RBC; but as there are several types of immature RBC a separation would be necessary before enzyme activity could be correlated with the stage of cell development. Sodium and potassium concentrations in the RBC of the various animals and patients exhibited no consistent relationship with the PPFase activity; hence it seemed unlikely that the cation concentration influenced the level of enzyme activity.

Introduction

The first paper in this series (8) described the determination of pyruvic phosphoferase (PPFase) activity in the erythrocytes of six mammalian and one avian species under normal conditions. The enzyme was activated by K^+ and inhibited by Na^+. The present paper records the alterations in PPFase activity that were observed in various experimental and disease states, viz., anemic rabbits and rats, tumor-bearing rats, and human patients with various blood disorders. The erythrocyte sodium and potassium concentrations were also determined, because of the known effects of these ions upon the enzyme activity.

Methods

Fresh specimens of heparinized blood were used to determine PPFase activity of the erythrocytes by the method previously described (8). Potassium chloride and magnesium sulphate were added to the undialyzed hemolyzates to give final concentrations of 0.10 and 0.005 *M* respectively. One unit of enzyme activity corresponded to the liberation of 1 μM. of pyruvate in 10 min. at 37° C. The activity was calculated in units per 10^9 cells (mean erythrocyte activity) or per milliliter of packed erythrocytes (enzyme concentration). Hematocrit determinations were carried out by centrifuging the whole blood for 30 min. at 3000 r.p.m. in a clinical centrifuge. Erythrocyte counts were made by the standard method and reticulocyte counts by the method of

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Brecher (1). Sodium and potassium determinations were made on red cells that had been washed twice in the centrifuge with 0.3 *M* sucrose solution and hemolyzed with 200 times their volume of water. The hemolyzates were sprayed directly into the flame of a Beckman model 9200 Flame photometer equipped with the acetylene burner and a photomultiplier attachment (2). The standard solution for these determinations contained 10 meq. of sodium and 100 meq. of potassium per liter.

Results

Anemia in Rabbits

A hemolytic anemia was produced in rabbits by parenteral injection of a 1% solution of acetylphenylhydrazine in warm 1% sodium chloride solution at a dose level of 20 mgm./kgm. body weight. Fig. 1 illustrates the changes that were observed in the erythrocytes of one rabbit during the development of the anemia and the subsequent recovery. The PPFase activity, whether it was expressed per cell or per milliliter of cell volume, paralleled the changes in mean erythrocyte volume. It appears that the immature erythrocytes (those with the greater cell volume) have a much higher PPFase activity than the normal cells.

This experiment was repeated on another rabbit, but the animal died on the eighth day. In this case reticulocyte counts and sodium and potassium determinations were also made, and the results are given in Table I. The

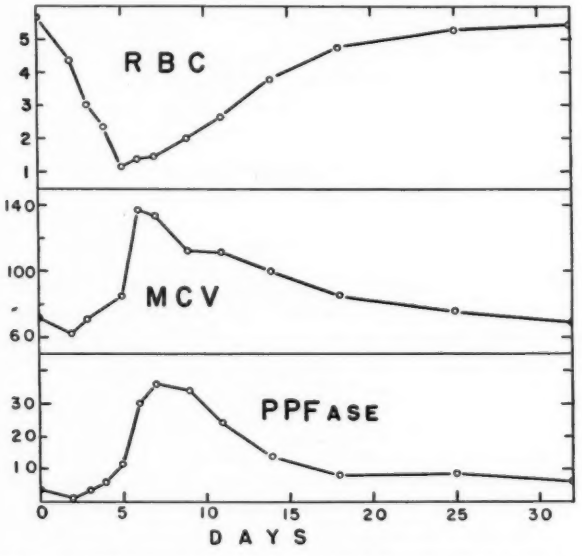

FIG. 1. Changes in the erythrocytes of a rabbit following administration of a single dose of acetylphenylhydrazine at a level of 20 mgm./kgm. body weight. (For abbreviations see footnote to Table I.)

enzyme activity paralleled the reticulocyte count, again indicating the association of high enzyme activity with the immature erythrocytes. As the anemia developed, the cell sodium increased while the potassium decreased. Later the distribution of these ions returned to normal.

Table II shows the effects of repeated injections of acetylphenylhydrazine (six injections over a period of 18 days) into a rabbit. Although the percentage of reticulocytes steadily increased, the PPFase activity showed striking variations, and in one blood sample no activity was measurable. Alterations in the cell sodium seemed to be correlated with the enzyme activity, as the sodium was greater in concentration when the enzyme activity was low.

The very low enzyme activities that were observed on certain occasions might have been due to the presence of an enzyme inhibitor formed from the

TABLE I

THE EFFECT OF ACETYLPHENYLHYDRAZINE INJECTION UPON THE ERYTHROCYTE PPFASE ACTIVITY OF A RABBIT

Time after injection, days	RBC	MCV	Retics.	PPFase		K	Na
				Per 10^9 cells	Per ml.		
0	5.48	68	2	5.6	82	95	9.0
1	4.74	79	—	6.8	86	69	14.9
2	3.80	59	6	3.5	59	59	23.7
3	2.48	67	18	6.9	103	63	25.7
5	1.40	97	58	28.2	290	98	14.0
7	1.48	132	70	52.0	393	97	5.0
8	Died						

In all tables:
RBC = erythrocyte count, in millions per μl.
MCV = mean cell volume, in μ^3.
Retics. = reticulocyte count, in % of the total erythrocytes.
PPFase = enzyme activity in μM. pyruvate liberated in 10 min. at 37° C., per 10^9 cells or per ml. of packed cells.
K, Na = meq. per liter of packed cells.

TABLE II

THE EFFECT OF REPEATED INJECTIONS OF ACETYLPHENYLHYDRAZINE (ACPH) ON THE ERYTHROCYTE PPFASE OF A RABBIT

Date	ACPH, mgm.	RBC	MCV	Retics.	PPFase		K	Na
					Per 10^9 cells	Per ml.		
May 31	45	6.85	59	3	3.3	56	92	11.1
June 2	30	5.30	61	5	2.8	46	83	14.4
June 4	0	2.70	71	25	1.0	14	78	20.7
June 7	25	2.10	111	40	9.0	81	101	8.5
June 9	30	2.72	112	55	0.0	0	101	13.3
June 11	0	3.10	89	60	4.6	52	103	9.0
June 15	35	3.60	93	80	0.5	5	100	11.6
June 17	40	3.00	85	95	3.0	35	112	15.4

drug. However, another experiment on repeated blood loss in a rabbit (nine bleedings totalling 185 ml. over 13 days) showed similar but not so marked variations. In this animal the mean cellular PPFase had dropped to 0.2 units within 17 days after the beginning of the experiment, although 10% reticulocytes were present. The variations in the cations were not so marked as in the previous experiment, and in this case a high sodium concentration was associated with high enzyme activity.

Anemia in Rats

Blood was taken at intervals from two groups of rats by heart puncture under ether anesthesia. On the first day 6 ml. was withdrawn from each animal, and this was followed by 2 ml. on each of the subsequent days recorded in Table III. The four animals in Group *A* each received daily injections of 0.062 μgm. of vitamin B_{12} in 0.1 ml. of saline, while the four animals in control Group *B* each received the same volume of saline alone.

Table III records the average values obtained for each group of rats. The variations in erythrocyte PPFase were analyzed statistically by the *t* test according to Fisher (4). The increase in PPFase was compared with the initial value and the probability is given in the table under *P*. The increase was considered statistically significant at the 5% level. In control Group *B* the blood loss did not produce a marked anemia and the mean cellular PPFase did not vary significantly from the initial level. In Group *A*, which received vitamin B_{12}, significant increases in enzyme activity were observed on the 3rd and 14th days. However, in the two groups there were no marked variations in the mean cell volume or the percentage of reticulocytes. There was no apparent correlation between the enzyme activity and the cellular sodium or potassium concentrations.

TABLE III

THE EFFECT OF REPEATED BLEEDING UPON THE PPFASE ACTIVITY OF RAT ERYTHROCYTES

The values are averages for each group of four rats. Group *A* received daily injections of 0.062 μgm. of vitamin B_{12}, and Group *B*, injections of saline only

Date	RBC	MCV	Retics.	PPFase		*P*	K	Na
				Per 10^9 cells	Per ml.			
Group A								
May 4	9.3	52	3	6.6±0.95	127	—	101	3.3
May 7	7.3	58	12	17.1±2.1	294	0.02	92	3.1
May 11	7.8	52	13	7.1±1.13	137	>0.05	100	3.0
May 18	9.4	51	7	12.0±0.56	235	0.02–0.05	90	3.9
Group B								
May 7	9.9	54	2	9.0±0.96	166	—	95	3.3
May 10	6.9	53	9	8.3±0.24	157	>0.05	105	4.7
May 14	7.9	52	13	7.5±0.72	145	>0.05	103	3.8
May 21	8.9	55	8	9.7±1.28	176	>0.05	92	3.0

The erythrocytes of rats bearing the Walker carcinoma 256 were examined because these animals are usually anemic. The results obtained with eight rats are summarized in Table IV. Not all the animals were severely anemic, but all had an abnormally high level of reticulocytes.

The mean erythrocyte PPFase activity for normal rats was previously found to be 7.2 ± 0.51 μM./10^9 cells (8). The standard deviation (S.D.) was 2.1 and we have taken the normal range to equal the mean $\pm$ 2 S.D. or 3.0–11.4 units. In every animal but one the mean cellular PPFase activity was elevated above normal. The activity per milliliter of packed cells was also high, but these values were not subjected to statistical analysis. The increase in enzyme activity roughly paralleled the degree of reticulocytosis, and the presence of these immature red cells was presumably a response to the anemia. There is no evidence that the presence of the tumors was responsible for the elevated PPFase activity. There was no correlation between the PPFase levels and the sodium or potassium concentrations.

TABLE IV

PPFase activity of erythrocytes of rats with Walker carcinoma 256

Rat No.	Wt. of tumor, gm.	RBC	MCV	Retics.	PPFase		K	Na
					Per 10^9 cells	Per ml.		
1	35	6.00	60	25	20.0	333	112	9.8
2	45	3.90	67	82	28.3	422	113	7.6
3	62	4.10	68	85	29.0	426	113	17.9
4	36	5.00	64	48	25.0	390	111	11.9
5	25	6.75	78	19	14.4	185	90	12.8
6	65	4.80	67	35	15.7	234	99	8.0
7	100	2.85	70	93	19.1	273	112	10.0
8	60	7.70	50	14	5.0	100	112	3.1

Human Patients with Anemia

The average value for the PPFase activity of human erythrocytes had been found to be 11.3 ± 0.52 units per 10^9 cells, with a standard deviation of 2.1 (8). The normal range was taken as equal to the mean $\pm$ 2 S.D. or 7.2–15.5 units. The PPFase activity of the erythrocytes of several normal persons was followed over a period of six to seven months and the maximum variation was about ± 1 unit, or about $\pm 10\%$ of the mean value.

Blood specimens from eight cases of pernicious anemia were examined. Patient U-1 was a newly diagnosed case and had not received any previous treatment. The others were patients who had returned to hospital for treatment of a recurrence of the anemia. Table V shows that in five cases the mean cellular PPFase was above the normal range. The PPFase activity did not seem to be correlated with the reticulocyte count, the mean cell volume, or the sodium and potassium concentrations.

TABLE V

PPFase activity of erythrocytes of pernicious anemia patients

Patient U-1 was a new case of pernicious anemia. All others were previously diagnosed cases whose symptoms had recurred. "Previous treatment" refers to treatment immediately before the blood samples were obtained

Case No.	Age, years	Sex	RBC	MCV	Retics.	PPFase		K	Na	Previous treatment
						Per 10^9 cells	Per ml.			
U-1	66	M	1.11	103	3	11.0	107	89	10.5	None
U-14	70	M	3.96	98	8	21.4	218	99	6.3	Vit. B_{12} 1 week
G-1	97	F	1.85	92	18	36.0	391	92	5.6	Transfusions
RA-1	73	F	1.05	129	6	25.0	194	79	13.7	Travert, glucose
RA-3	75	M	1.78	143	4	11.0	77	67	14.1	Liver ext., penicillin
RA-7	59	F	0.89	112	2	21.6	193	—	—	Transfusions, B_{12}, thiomerin
RA-4	76	M	1.60	128	16	13.9	109	95	11.3	?
M-3	57	F	1.00	150	0	22.0	147	—	—	None

Fig. 2 illustrates the changes that were observed in the erythrocytes of case U-1, following daily injections of 30 μgm. of vitamin B_{12}. A striking increase in PPFase activity (per cell and per milliliter of packed cells) may be noted; this increase paralleled the percentage of reticulocytes and the mean cell volume. The potassium concentration increased while the sodium decreased during the treatment.

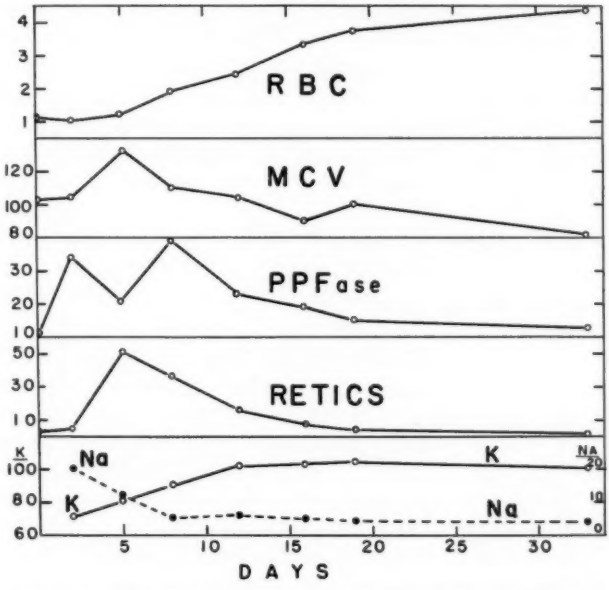

FIG. 2. Changes in the erythrocytes of a pernicious anemia patient during treatment with daily injections of 30 μgm. of vitamin B_{12}. (For abbreviations see footnote to Table I.)

The results obtained with a group of patients suffering from various other types of anemia are given in Table VI. Five cases showed a PPFase activity above the normal range, but again there was little relationship with the degree of reticulocytosis. An exceptionally high level of PPFase was noted in patient M-4, a case of microcytic nutritional anemia. Case G-2, aleukemic myelosis, was treated with one transfusion daily over a period of 13 days. The PPFase activity remained constant while the erythrocyte count rose from 1.9 to 3.8 millions and the percentage of reticulocytes dropped from 15 to 7.

Table VI also includes a few cases of various disease conditions, none of which was markedly anemic. Only one patient (case G-5, carcinoma of the ovaries) had an enzyme level above the normal range. In every case the reticulocyte count was normal and the erythrocyte sodium and potassium showed no marked deviations.

TABLE VI

PPFase activity of erythrocytes of anemic patients and patients with various diseases

Case No.	Diagnosis	Age, years	Sex	RBC	MCV	Retics.	PPFase		K	Na
							Per 10^9 cells	Per ml.		
*Anemic patients**										
U-2	Pseudohemophilia	0.5	M	2.35	101	3	15.7	155	94	4.8
U-3	Congenital hemolytic anemia	1.2	M	2.72	88	39	12.6	143	81	10.4
G-2	Aleukemic myelosis	40	F	1.90	92	15	19.3	209	90	5.4
G-3	Uterine bleeding	27	F	2.86	86	3	6.0	70	—	—
RA-2	Aplastic anemia	5	M	3.90	87	0	5.8	67	87	8.2
M-1	Hypochromic normocytic anemia	68	F	2.80	100	2	22.5	225	83	12.5
M-4	Nutritional anemia	46	F	3.88	48	2	26.0	542	81	8.7
M-5	Hemorrhagic anemia	77	F	2.60	84	7	17.8	211	96	3.9
M-6	Acute lymphatic leukemia	24	F	1.80	50	6	15.4	308	—	—
Patients with various diseases										
M-2	Hepatitis	41	F	4.80	88	1	14.5	165		
B-1	Diabetes	22	M	4.88	—	1	11.0	—		
m-2	Uremia	52	M	3.15	95	—	13.0	137		
G-4	Uremia	49	F	3.40	75	2	14.0	186		
m-4	Pyelitis	33	F	3.00	—	—	7.3	—		
G-5	Carcinoma of ovaries	51	F	3.25	68	3	28.0	412		
RA-5	Carcinoma of stomach	67	F	4.58	93	—	6.9	74		
RA-6	Carcinoma of stomach	75	M	3.50	87	3	14.5	166		
M-3	Gastric ulcer	81	F	3.36	95	—	12.1	127		
M-7	Acute myeloid leukemia	—	M	4.00	39	3	6.3	161		

* *All these patients had received numerous blood transfusions.*

Discussion and Conclusions

In the previous paper of this series (8) it was shown that the immature erythrocytes of the young rat had an extremely high PPFase activity. In the present paper we have shown that anemia in animals and in human patients is usually associated with a high erythrocyte PPFase activity. This can presumably be attributed to the new erythrocytes thrown into the circulation as a response to the anemia; however, the enzyme activity did not always parallel the percentage of reticulocytes or the mean cell volume. (The newer red cells have a greater mean volume than the mature cells.)

Repeated injection of acetylphenylhydrazine and repeated blood loss in rabbits caused marked fluctuations in the PPFase activity. It is possible that the very low values that were observed may be due to depletion of some precursor for the enzyme. Further investigation is required to explain these variations.

It is of interest that an increase in enzyme activity accompanied the administration of vitamin B_{12} to a group of rats that was subjected to repeated blood loss, and to a pernicious anemia patient. In the pernicious anemia patient the increase in PPFase activity did accompany a rise in the proportion of reticulocytes and in the mean cell volume.

On the whole, the results of the erythrocyte sodium and potassium determinations exhibit no very definite relationship with the enzyme activity. In one experiment with a hemolytic anemia in a rabbit (Table I) there was an initial decrease in potassium and increase in sodium; this was followed by an increase in potassium and decrease in sodium that paralleled the increasing PPFase activity. In the experiment recorded in Table II (repeated administration of acetylphenylhydrazine to a rabbit) the sodium concentration varied inversely with the enzyme activity. In the treatment of a case of pernicious anemia with vitamin B_{12} (Fig. 2) the cell potassium increased and sodium decreased as new red cells entered the circulation. Kerr (6) and Henriques and Ørskov (5) have reported that erythrocyte regeneration following blood loss in animals is accompanied by increased erythrocyte potassium and decreased sodium. Thus there is evidence that the ratio K/Na is higher in the immature red cell than in the normal cell. But in most of our determinations there was no obvious relationship between the cation concentrations and the PPFase activity or the age of the cells. It is not possible to draw any conclusion regarding a causal relationship between enzyme activity and cation concentration.

It seems clear that the PPFase activity and the cation distribution cannot be related to any one type of erythrocyte in the circulation. Davis, Bigelow, and Alpen (3) have recently reported that in the anemic rat only a small proportion of the new red cells is made up of reticulocytes; other large but non-reticulated red cells are present. Seno and his co-workers (7) have distinguished various types of erythrocytes by cytochemical and morphological methods. Thus there is a mixed population of erythrocytes of varying ages

in the blood of anemic animals, and this adds to the difficulty of interpreting "mean cellular" measurements. On the other hand, some method of separation of red cells of different ages could afford a unique opportunity for studying the metabolic patterns of cells at different stages of maturity.

Acknowledgments

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THE METABOLISM OF NORMAL BRAIN AND HUMAN GLIOMAS IN RELATION TO CELL TYPE AND DENSITY[1]

By Irving H. Heller[2] and K. A. C. Elliott

Abstract

Per unit weight, cerebral and cerebellar cortex respire much more actively than corpus callosum. The rate per cell nucleus is highest in cerebral cortex, lower in corpus callosum, and still lower in cerebellar cortex. The oxygen uptake rates of the brain tumors studied, with the exception of an oligodendroglioma, were about the same as that of white matter on the weight basis but lower than that of cerebral cortex or white matter on the cell basis. In agreement with previous work, an oligodendroglioma respired much more actively than the other tumors. The rates of glycolysis of the brain tumors per unit weight were low but, relative to their respiration rate, glycolysis was higher than in normal gray or white matter. Consideration of the figures obtained leads to the following tentative conclusions: Glial cells of corpus callosum respire more actively than the neurons of the cerebellar cortex. Neurons of the cerebral cortex respire on the average much more actively than neurons of the cerebellar cortex or glial cells. Considerably more than 70% of the oxygen uptake by cerebral cortex is due to neurons. The oxygen uptake rates of normal oligodendroglia and astrocytes are probably about the same as the rates found per nucleus in an oligodendroglioma and in astrocytomas; oligodendroglia respire much more actively than astrocytes.

Introduction

The absence of information about the relative metabolic activities of neuronal and non-neuronal brain elements is such a deficiency in our knowledge of brain metabolism that information obtained even with abnormal tissue derived from non-neuronal brain cells could be valuable. In the following study, therefore, the respiratory activities per unit weight and per cell of normal cerebral cortex, of cerebellar cortex (which contains many more neurons per unit weight), of corpus callosum (which contains almost as many nuclei as cerebral cortex (6) but no neurons), and of human glial tumors have been compared.

Figures for the respiratory and glycolytic activity of a large number of tumors, including a number of human tumors, are listed in the preface to Dickens' translation of Warburg's monograph on tumor metabolism (12). Many further figures have been published but, as far as we are aware, only one study of the metabolism of brain tumors, by Victor and Wolf (11), has appeared. It seemed of interest to obtain further data on such tumors and to relate metabolic activity to fresh weight, to dry weight, and, in view of the variability in cellularity among tumors of similar type, to cell density.

Methods

The human material was excised *en bloc* during neurosurgical operations. Cats were decapitated with a large double-action tree pruner; dogs, anesthe-

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Contribution from the Donner Laboratory of Experimental Neurochemistry, Montreal Neurological Institute, and the Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec.

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tized with chloralose-urethane, were killed by bleeding or by occlusion of the trachea at the conclusion of experiments not connected with this study. The brains were removed immediately and the tissue was sliced by means of a Stadie–Riggs (9) microtome, without moistening, in a humid chamber. The microtome was designed to cut slices about 0.5 mm. thick, but for corpus callosum and occasionally with other slowly respiring tissues the microtome was adjusted to cut slices about 0.75 mm. thick. Slices were weighed on a torsion balance before introduction into the manometer vessels. Other slices were weighed and then dried at 110° to establish the wet weight/dry weight ratio. Remaining tissue was stored at − 20° and, when convenient, cell densities were estimated by determinations of the total desoxyribonucleic acid (DNA) content and the DNA per nucleus as described in a previous paper (6). Except for the two determinations on human white matter, the cell densities mentioned in this paper were all recorded in the previous publication.

Oxygen consumption, respiratory carbon dioxide evolution, and aerobic glycolysis were measured in duplicate in bicarbonate-buffered medium by means of the Summerson modification (10) of the Dixon-Keilin apparatus at 38°. The medium, which resembled spinal fluid, contained the following, in millimoles per liter: NaCl 122, KCl 3, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 10. The vessels contained 95% oxygen and 5% carbon dioxide. The experimental period was usually 90 min. Relatively large amounts of tissue, up to 200 mgm. per vessel, were used whenever possible in order to obtain large readings and to minimize the effect of blank errors. The thermobarometer vessel contained a piece of filter paper in the inset as a control for the small evolution of gas which has been found to occur in vessels, containing medium and paper but no tissue, which have been filled with oxygen – carbon dioxide mixture and equilibrated in the usual way. Anaerobic glycolysis was measured in duplicate in the same medium in ordinary Warburg vessels filled with oxygen-free nitrogen containing 5% carbon dioxide; a separate thermobarometer containing no paper was used. Oxygen consumption measurements in phosphate-buffered medium were carried out in standard Warburg vessels with alkali papers in the insets. The medium had the same composition as the bicarbonate-buffered medium except that 17.5 mM. phosphate replaced the bicarbonate and calcium was omitted. The pH of this medium was originally 7.8 but fell to about 7.2 by the end of experiments. The vessels were filled with pure oxygen.

At the end of aerobic experiments with tumor tissues, the slices from the experimental vessels were fixed in formalin, stained, and examined histologically.

Results and Discussion

Normal Brain Tissues

In Table I oxygen uptake rates, per unit weight and per nucleus, of three widely different types of normal brain are compared. (Determinations were

done in calcium-free phosphate-buffered medium or in complete bicarbonate-buffered medium as noted. Respiration is usually more active in the former than in the latter medium.) Since fresh normal human brain tissues become available rarely and not in sufficient amount for all the necessary determinations, comparisons will be based mainly on results obtained with dog and cat brain. On the weight basis cerebellar cortex respires somewhat less actively than cerebral cortex. Previous workers have obtained rates for cerebellar cortex which were slightly higher than (1, 5), or equal to (13) the rate for cerebral cortex from the same species. Corpus callosum respires considerably less actively. The respiration rate per cell is, however, highest in the cerebral cortex and lowest in cerebellar cortex.

TABLE I

OXYGEN UPTAKE OF NORMAL BRAIN TISSUES

Tissue	Nuclei per cu. mm., thousands	Oxygen uptake rate, μl. per hr. Per mgm. fresh wt. Range	Per mgm. fresh wt. Mean	Per mgm. dry wt.*, mean	Per 10^6 nuclei, mean	Medium†	Number of samples/animals
Cat							
Cerebral cortex	128		1.7	8.8	13	B	2/2‡
" "	"	2.1 –2.7	2.4	12.4	19	P	20/4‡
Cerebellar cortex	808	2.0 –2.2	2.1	10.6	2.6	P	4/2
Corpus callosum	135	0.55–0.80	0.67	2.2	5.0	B	4/2
" "	"	0.65–0.90	0.77	2.5	5.7	P	4/2
Dog							
Cerebral cortex	148		1.4	8.5	9.5	B	2/2‡
" "	"	1.5 –2.6	2.15	13.1	14.5	P	8/4‡
Cerebellar cortex	568	1.6 –1.8	1.7	10.8	3.0	P	4/2
Corpus callosum	145	0.6 –0.8	0.69	2.2	4.8	P	4/2
Human							
Cerebral cortex	131	0.7 –1.3	1.2	6.7	(9.2)§	B	8/8‡
" "	"	1.4 –2.3	1.9	10.6	(14.5)§	P	12/12‡
Temporal white matter			0.75	2.7	(6.7)**	P	1/1
Corpus callosum	112						
Cerebellar white matter	42	0.23–0.45	0.33	1.2	7.9	P	4/1

** Initial dry weight. From the fresh weight of tissue in vessel and the fresh weight/dry weight ratio determined on a separate sample.*

† B, bicarbonate-buffered medium; P, calcium-free phosphate-buffered medium.

‡ From Elliott et al. (3, 4) and further figures obtained in the present study.

§ The estimate of nuclei was on parietal cortex while the oxygen uptakes were determined on mixed areas of cortex, mostly from epileptic patients.

*** Assuming the same nuclear density as in corpus callosum.*

The respiration per cell of corpus callosum, which contains no nerve cell bodies, is more active than that of cerebellar cortex, in which the large majority of the cells are neurons. Non-neuronal, presumably glial, cells in the brain can, therefore, respire considerably more rapidly than some neurons. The possibility that the axons in corpus callosum contribute considerably to the

total metabolism of corpus callosum seems unlikely since the metabolism of peripheral nerve is very low. Oxygen uptake rates found for sciatic nerve in the phosphate medium were 0.29 and 0.25 (cat) and 0.27 (rabbit) μl. per mgm. fresh weight per hr.

The high rate per nucleus in cerebral cortex suggests either that many neurons in this part of the brain respire much more rapidly than most of the neurons in cerebellar cortex or that the non-neuronal cells in cerebral cortex are more active than would be expected from the results obtained with corpus callosum. The former alternative is more probable since the cell bodies of the neurons of cerebral cortex are larger in volume, many being 10 or more times larger, and the volume of their processes is considerably greater, than most of those in cerebellar cortex. Lowry *et al.* (8) have evidence that the metabolism of dendrites is probably high.

Cerebral cortex is the tissue most commonly used in studies of brain metabolism *in vitro*. But it has long been uncertain to what extent measurements of oxygen uptake, and of other metabolic processes, of cerebral cortex reflect neuronal metabolism rather than the activity of non-neuronal cells which constitute a large majority of the cells in this tissue. If the glial cells of cerebral cortex respire at the same average rate as those in corpus callosum and constitute five-sixths[3] of the total number of cells in cerebral cortex, it can be calculated from the mean figures in Table I that glial cell respiration could account for only about 28% (dog) or 25% (cat) of the cortical respiration. The respiration by neurons must, therefore, account for over 70% and the average respiration of neurons in cerebral cortex must be about 63 (dog) or 85 (cat) μl. per 10^6 neurons. Similar figures are indicated by the determinations on human brain.

The results obtained with tumors by ourselves and Victor and Wolf, see below, suggest that astrocytes respire much less actively than oligodendroglia. Though we know of no actual counts, histological observations indicate that cerebral cortex contains a much greater proportion of astrocytes to oligodendroglia than does corpus callosum so that the average respiration rate of glial cells would be lower in cerebral cortex than in corpus callosum. It is probable, therefore, that glial cells contribute still less to the total respiration of cerebral cortex than is calculated from the average respiration per nucleus in corpus callosum. The respiration rate per neuron would then be higher than calculated above and the percentage of the respiration accounted for by neurons in cerebral cortex may be considerably greater than 70%. These arguments assume, as seems probable, that other cell types, such as microglia and perivascular cells, contribute little to the total metabolism.

Gliomas

The low respiratory activity and the inhomogeneity of many of the tumors decreased the accuracy with which the oxygen uptake and, especially, the

[3] *This figure is the estimate of Dr. Jerzy Olszewski, Department of Neuroanatomy, Montreal Neurological Institute (personal communication).*

respiratory carbon dioxide evolution could be determined in the bicarbonate-buffered medium, since the Dixon-Keilin procedure depends upon having equal samples of tissue in the control and experimental vessels. Two extreme examples illustrate the uncertainties. With one active astrocytoma which appeared grossly homogeneous, relatively high anaerobic glycolysis was found but no oxygen uptake or aerobic glycolysis. The slices used for the aerobic determinations were found histologically to be completely necrotic. With a calcified tumor "impossible" h_1 readings, upon which the measurement of carbon dioxide evolution depended, were obtained. Some solid calcified material associated with the tumor was ground up and was shown manometrically to contain about 7% of carbonate. Different amounts of calcified material in the control and experimental slices would have accounted for the "impossible" h_1 readings.

In Table II results of determinations of oxygen uptake rates of brain tumors are summarized. The rates of oxygen uptake of the tumors were very variable but in all cases, except with one sample from an oligodendroglioma, the rates per unit of fresh weight were only one eighth to one half of the average for human cerebral cortex, and lower than rates reported (7) for a number of non-nervous human tissues, but in the range found for human white matter. One sample from an oligodendroglioma gave a rate higher than the average for cerebral cortex. Unfortunately only one of these rare tumors has become available to us in nearly two years and another sample from this tumor contained much calcified material. The high respiration rate obtained with the one active sample, however, confirms the similar finding of Victor and Wolf (11) with two tumors of this type.

The fresh weight/dry weight ratio for most of the tumors was about 6 but two astrocytomas with very high moisture content gave ratios of 14. On the dry weight basis most of the tumors, other than the oligodendroglioma on which the dry weight was not determined, respired at about one third to one half the rate of cerebral cortex. Per nucleus their respiration rate was relatively still lower than that for cerebral cortex and considerably lower than that for white matter. The rate per nucleus of even the actively respiring oligodendroglioma sample was lower than the rate per nucleus of cerebral cortex but about the same as for human white matter (allowing for the fact that the former was measured in the bicarbonate-buffered medium and the latter in the calcium-free phosphate medium).

Results of determinations of respiratory quotient (R.Q., ratio of respiratory carbon dioxide evolution to oxygen uptake) and glycolytic activity are summarized in Table III. Though inaccuracy precludes a definite conclusion about R.Q. values, most of the more reliable figures were high and the range was about the same as was reported by Elliott and Baker (2) for transplanted rat tumors and for a number of normal tissues.

The glycolytic activity, like the respiratory activity, of the brain tumors studied was low. It seems unlikely, therefore, that acidity resulting from lactic acid formation can contribute appreciably to the disturbance of brain

TABLE II

OXYGEN UPTAKE OF HUMAN GLIOMAS

Patient	Tissue	Nuclei per cu. mm., thousands	Oxygen uptake rate, μl. per hr.			
			Per mgm. fresh wt.	Per mgm. dry wt.*	Per 10^6 nuclei	Medium†
I.F.	Medulloblastoma	1170	0.56, 0.47	3.4, 2.9	0.48, 0.40	B
G.F.	Medulloblastoma	1600	0.37, 0.32	2.4, 2.1	0.23, 0.20	P
E.B.	Glioblastoma	740	0.49, 0.42		0.64, 0.57	B
P.B.	Glioblastoma	380	0.51, 0.50, 0.39	3.7, 3.6, 2.9	1.3, 1.3, 1.0	P
M.M.	Malignant glioma, necrotic	400	(0.32)	(2.0)	(0.8)	B
W.S.	Astrocytoma	170	0.24, 0.17	3.4, 2.5	1.4, 1.0	B
C.J.	Astrocytoma	210	0.20, 0.17	2.9, 2.5	0.95, 0.83	B
L.C.	Astrocytoma	420	0.43	2.6	1.0	B
Y.C.	Astrocytoma, calcified	260	0.16	1.2	0.6	B
D.M.	Oligodendroglioma, calcified	298	1.4, (0.4)		4.7, (1.3)	B

Figures in parentheses are somewhat doubtful owing to much necrosis or calcification.

* *As in Table I.*

† *As in Table I.*

TABLE III

RESPIRATORY QUOTIENT, AEROBIC AND ANAEROBIC GLYCOLYSIS IN HUMAN BRAIN TUMORS AND NORMAL BRAIN

Patient	Tissue	R.Q.	Aerobic glycolysis*		Anaerobic glycolysis	
			μl. per hr. per mgm. fresh wt.	A/O_2†	μl. per hr. per mgm. fresh wt.	A/O_2†
	Human gliomas					
I.F.	Medulloblastoma	0.98, 0.87	0.38, 0.30	0.81, 0.54	1.32, 1.15	2.4
E.B.	Glioblastoma	0.7 ±	0.21 ±	0.5 ±	0.72, 0.60	1.4
M.M.	Malignant glioma, necrotic	(0.7 ±)	(0.69)	(2.2)	0.58, 0.51	(1.7)
W.S.	Astrocytoma	0.86, 0.72	0.18, 0.18	1.05, 0.75	0.42, 0.26	1.7
C.J.	Astrocytoma	1.0, 0.92	0.19, 0.16	1.0, 0.9	0.32, 0.29	1.6
L.C.	Astrocytoma	0.89	0.28	0.66	0.83, 0.52	1.6
M.S.	Astrocytoma				0.57, 0.48	
Y.C.	Astrocytoma, calcified		0.3 ±	2.0 ±	0.42, 0.36	2.4
D.M.	Oligodendroglioma, calcified	1.06	0.2 ±	1.4 ±	0.74, 0.52	0.45
	Normal brain					
	Human cerebral cortex‡	0.92 (av.)	0.4 to 0.8	0.3 to 0.6	1.3, 2.6	1.1, 2.2
	Human corpus callosum				0.34, 0.31	
	Cat cerebral cortex‡	1.0, 0.95	0.48, 0.35	0.2 and 0.3	1.4, 1.3	0.8 to 1.4
	Cat corpus callosum§	0.89 (av.)	0.21 (av.)	0.31 (av.)	0.38 (av.)	0.55 (av.)

* *Figures give the average rate over 90 min. The rates with normal brain are higher initially and fall off.*
† *Ratio of the mean rate of acid formation (glycolysis) to the mean rate of oxygen uptake.*
‡ *From Elliott et al. (3, 4).*
§ *Averages of four determinations (two animals).*

function caused by these tumors. The figures, which ranged from 0.2 to 0.4 μl. per mgm. of fresh weight for aerobic glycolysis and from 0.3 to 1.3 for anaerobic glycolysis, are lower than those obtained with cerebral cortex but of the same order as, or higher than, the very low activity of white matter.

Warburg (12) and others have observed that anaerobic glycolytic activity is high in most tumors and in certain normal tissues including cerebral cortex. Though the absolute rates were low in these gliomas, the anaerobic glycolytic activity was high relative to their respiratory activity. The ratios of anaerobic glycolysis to oxygen uptake rate were mostly in the same range as in cerebral cortex and higher than in corpus callosum.

High aerobic glycolysis is a characteristic, but not exclusive, property of tumor tissues. Relative to their oxygen uptake rates, most of these brain tumors showed fairly active aerobic glycolysis which was higher than was found with cerebral cortex or corpus callosum. The rate was well maintained whereas it usually falls of rapidly in cerebral cortex or corpus callosum while the respiration rate remains nearly constant.

In their study of brain tumor metabolism Victor and Wolf (11) used small samples obtained at biopsy, and micro equipment, and measured the aerobic and anaerobic activities in different media. The oxygen uptake rates found by them for medulloblastoma, glioblastoma, and astrocytoma were in general even lower, sometimes considerably so, than the rates here reported. But with two samples of oligodendroglioma, they obtained rates nearly as high as our higher figure for this tumor. Glycolysis rates in the few comparable cases were about the same as or higher than ours, and definitely higher relative to the oxygen uptake rates. They gave only three figures for R.Q. which cover the range of those here reported. Great variability is apparent in all activities measured by Victor and Wolf as by ourselves.

The ratios of the respiratory activity, per nucleus, of cerebral cortex to those of cerebellar cortex and corpus callosum of cat and dog are fairly similar (Table I). If about the same ratio holds for the human brain tissues we would expect the respiration per 10^6 nuclei in human corpus callosum to be about one third of that in cerebral cortex, namely about 4.8 μl. per hr. in the phosphate medium. The approximate mean figure obtained with human white matter was about 7. The rate per nucleus of the active oligodendroglioma, 4.7, was close to these figures. (It was determined in the bicarbonate medium and would have been nearer 7 in the phosphate medium.) This fits in with the impression that the majority of cells in corpus callosum are oligodendroglia. It suggests that normal oligodendroglia consume oxygen relatively rapidly like oligodendroglioma cells and that the malignant habit does not greatly change the rate of respiration per nucleus of glial cells. The rates found for our one sample of oligodendroglioma (together with the two reported by Victor and Wolf) and perhaps those for other gliomas may represent approximately the rates for normal oligodendroglia and astrocytes respectively. On the other hand, since the ratios of glycolysis to respiration are mostly elevated, glycolytic activity per cell does seem to be increased by the malignant transformation in the gliomas.

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THE AMPEROMETRIC DETERMINATION OF GLUTATHIONE REDUCTASE ACTIVITY IN HUMAN ERYTHROCYTES[1]

By H. Bruce Collier and Sheila C. McRae

Abstract

Glutathione reductase activity of hemolyzates of human erythrocytes was measured by an amperometric titration of the reduced glutathione that is formed from oxidized glutathione. The electron donor in the system was reduced triphosphopyridine nucleotide, produced by the glucose-6-phosphate dehydrogenase of the cells. Removal of the red-cell stromata from hemolyzates slightly increased the reductase activity. Addition of Na^+, K^+, or Ca^{++} had no effect on the enzyme. No marked inhibition was observed in the presence of phenothiazine, phenothiazone, phenylhydrazine, or *p*-chloromercuribenzoate.

Introduction[2]

Glutathione reductase catalyzes the reaction:

$$GSSG + 2TPNH \longrightarrow 2GHS + 2TPN^+.$$

This enzyme has been detected in pea seeds by Mapson and Goddard (11), in wheat germ by Conn and Vennesland (4), and in various tissues of the rat by Rall and Lehninger (13). Meldrum and Tarr (12) observed the enzymatic reduction of GSSG by rat erythrocytes in the presence of G-6-P and TPN^+, and Francoeur and Denstedt (6) recently reported the presence of the enzyme in human, rabbit, and rat erythrocytes. Benesch and Benesch (1) have stressed the importance of sulphydryl compounds in the maintenance of the integrity of the erythrocyte. Glutathione reductase evidently plays a role in the maintenance of the GSH in the reduced state.

Most workers have determined GSSG reductase activity by iodometric titration of the GSH that is formed. Francoeur and Denstedt (6) employed an indirect method in which GSH is measured through its influence upon the glyoxalase system of the erythrocyte. We proposed to investigate an amperometric titration method for the direct determination of GSH as it is formed by reductase activity.

Experimental

Principle of the Method

Glutathione reductase activity of erythrocytes was measured by a modification of the methods of Conn and Vennesland (4) and of Rall and Lehninger (13). In the presence of G-6-P, TPN^+ is reduced by the G-6-P dehydrogenase of the erythrocytes. The TPNH that is thus formed serves as the electron donor in the enzymatic reduction of GSSG. The GSH is then determined amperometrically.

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[2] *The following abbreviations are used:* GSSG, *oxidized glutathione;* GSH, *reduced glutathione;* TPN^+ *and* TPNH, *the oxidized and reduced forms, respectively, of triphosphopyridine nucleotide;* TRIS, *tris(hydroxymethyl)aminomethane;* EDTA, *ethylenediamine tetraacetate;* G-6-P, *glucose-6-phosphate.*

The erythrocyte G-6-P dehydrogenase was evidently present in excess, as further addition of this enzyme to the hemolyzates did not increase the observed reductase activity, and the reductase was therefore the limiting factor in this sequence of reactions. Rall and Lehninger (13) added nicotinamide to their assay system, but this was not found necessary with hemolyzates.

Materials

Glucose-6-phosphate was prepared as the barium salt by Mr. P. F. Solvonuk, and the barium was removed by treatment with potassium sulphate. TPN^+ "80" and G-6-P dehydrogenase were products of Sigma Chemical Co. GSSG and GSH were obtained from Schwarz Laboratories, Inc. TRIS buffers were prepared by addition of hydrochloric acid to solutions of tris-(hydroxymethyl)aminomethane. EDTA was a sample of Sequestrene from the Alrose Chemical Co.

Enzyme Assay

Erythrocytes from heparinized blood were washed three times in the centrifuge with 1% sodium chloride. A 0.25 ml. volume of the packed cells was hemolyzed in 2.0 ml. of water and to this were added the following (final concentration as given, in a total volume of 4.0 ml.): TRIS buffer, pH 7.4 (0.04 *M*), GSSG (0.001 *M*), G-6-P (0.002 *M*), TPN^+(10^{-4} *M*), magnesium chloride (0.01 *M*).

This mixture was incubated for 15 min. at 37° C. in a centrifuge tube, and at the end of that time 4.0 ml. of 10% trichloroacetic acid was added as deproteinizing agent. The mixture was centrifuged and the GSH in the supernatant fluid was determined. The initial GSH concentration was determined on unincubated control samples of the same composition as the incubated samples. GSSG reductase activity was expressed as the *increase* in GSH under these conditions, in mgm. per ml. of packed cells.

Glutathione Determination

In preliminary experiments the photometric nitroprusside method of Grunert and Phillips (7) for determination of GSH was applied to metaphosphoric acid filtrates. The method gave quantitative recovery of GSH added to hemolyzates, and GSSG reductase activity of human erythrocytes was demonstrated. However, it was desired to test the effect of various colored enzyme inhibitors upon the system, and the photometric method was therefore replaced by an amperometric titration of GSH.

Kolthoff and Harris (8) described an amperometric titration for mercaptans, in which a rotating platinum electrode is used, and the mercaptans are titrated with standard silver nitrate. Modifications of this method have recently been used for biological materials by Benesch and Benesch (1), Bidmead and Watson (2), Ling and Chow (10), Wald and Brown (15), and Weissman, Schoenbach, and Armistead (16). According to Ling and Chow (10) ergothioneine does not interfere in the titration of blood. Unfortunately the methods for titration of GSH and GSSG that have recently been described by Kolthoff and his co-workers (9, 14) were not available to us at the time.

We adopted the method of Bidmead and Watson (2) with minor modifications. The GSH was determined in 4.0 ml. of the protein-free solution. To this were added: 0.3 ml. of 0.003 *M* EDTA (to prevent oxidation of GSH by heavy metals in the alkaline solution), 21 ml. of 0.06 *M* ammonium nitrate, 4.0 ml. of absolute ethanol, and 0.3 ml. of concentrated ammonia to give a final pH of 8.5–9.0.

The rotating platinum electrode was attached to an Eberbach hollow-spindle stirrer; it was then connected in series with a galvanometer (0.3 μa. per scale division), a mercury – mercuric iodide reference electrode, and, through a salt bridge, to the solution to be titrated (8). The solution was titrated with 0.001 *N* silver nitrate and the galvanometer current was plotted against the number of milliliters added. As the end point was reached the current suddenly increased, and the exact end point was determined from the break in the titration curve (8). GSH when added to hemolyzates was recovered quantitatively (101–103%) by this procedure. In preliminary experiments with GSSG from several sources the recovery of GSH in the presence of GSSG was not quantitative; but this difficulty was not encountered when GSSG from Schwarz Laboratories was used.

Reduction of GSSG was found to be directly proportional to enzyme concentration, as illustrated in Fig. 1, and replicate assays agreed within 5%. For eight specimens of human blood the mean reductase activity was 9.1 ± 0.51 mgm. of GSH per ml. of packed cells, with a range of 6.8 to 11.0.

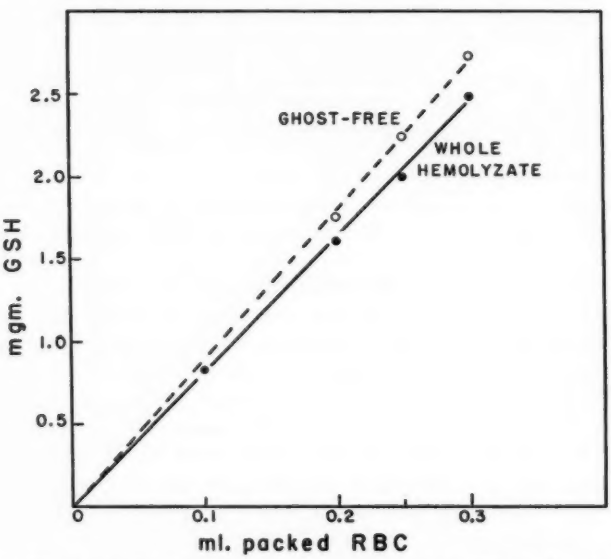

FIG. 1. The relationship between enzyme concentration (milliliters of packed erythrocytes per 2.0 ml. of hemolyzate in a total volume of 4.0 ml.) and GSSG reductase activity (mgm. of GSH formed in 15 min. at 37° C.). The solid line represents whole hemolyzate; the broken line, ghost-free hemolyzate.

Factors Affecting Enzyme Activity

Removal of the red-cell "ghosts" by centrifugation of the hemolyzates resulted in a slight increase in reductase activity, as seen in Fig. 1. For routine determinations of enzyme activity, however, whole hemolyzates were always used. It appears that the presence of the "ghosts" causes some inhibition of the reductase. It is interesting to note that Eggleton and Fegler (5) found the presence of the stromata to decrease the rate of GSH oxidation in hemolyzates of ox and horse erythrocytes; but it is not clear whether their finding is related to the inhibition of glutathione reductase activity which we observed.

The addition of Na^+, K^+, or Ca^{++} at 0.02 *M* final concentration had no effect on the reductase activity.

Phenothiazine, phenothiazone, phenylhydrazine, and *p*-chloromercuribenzoate all inhibit erythrocyte glyoxalase (3), which is a thiol enzyme. The effect of the first three of these compounds on the G-6-P dehydrogenase – GSSG reductase system was slight and irregular. Even *p*-chloromercuribenzoate, which is a powerful inhibitor of thiol enzymes, had little effect on the system: at a concentration of 10^{-4} *M* it produced only 20% inhibition. Hence the experiments with these compounds were not pursued further.

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THE RELEASE OF CORTICOTROPHIN BY ANTERIOR PITUITARY TISSUE *IN VITRO*[1]

By Murray Saffran and A. V. Schally

Abstract

The release of ACTH by rat anterior pituitary tissue *in vitro* was used as a test system for the detection of factors that stimulate ACTH-release. The results indicate that:

1. Epinephrine or arterenol, added by themselves, are without effect.
2. Hypothalamic tissue alone is also ineffective.
3. The combination of hypothalamic tissue with epinephrine or arterenol increases the release of ACTH about threefold.
4. Brain cortex can replace hypothalamus.
5. Liver cannot replace neural tissue; acetyl choline and serotonin cannot replace epinephrine or arterenol.
6. The greatest stimulation of ACTH-release (six- to eight-fold) occurs with posterior pituitary tissue plus arterenol. The arterenol may be replaced by hypothalamus or sphingosine, but not by dopamine (3,4-dihydroxyphenylethylamine), which is structurally similar to arterenol.
7. The posterior pituitary is probably involved in the response of the anterior pituitary–adrenocortical system to stress.

Contemporary theories suggest that the release of corticotrophin (ACTH) from the anterior pituitary is under humoral control. Three kinds of substances have been put forward as the controlling agents: epinephrine (2), circulating corticoids (8), and a hypothetical hypothalamic factor (1). The persistence of an increased release of ACTH after stress in adrenalectomized animals (11) rules out epinephrine of adrenal origin and corticoids as participating in anything but the fine adjustment of the release of ACTH. Therefore, the most tenable hypothesis is that of a hypothalamic factor. Recently, Slusher and Roberts (10) have claimed that they have obtained a lipid concentrate from bovine posterior hypothalamus tissue with some of the properties predicted for the hypothalamic factor. However, these authors used intact rats for the detection of the activity so that the evidence does not prove that their lipid material acts directly on the anterior pituitary.

Most workers in this field have used indirect indices of ACTH-release, such as eosinopenia and a decrease in adrenal ascorbic acid. McCann and Sydnor (4) were the first to measure blood and pituitary ACTH (by the method of Sayers *et al.* (9)) to demonstrate that hypothalamic lesions abolished the release of ACTH that usually follows stress. Moreover, McCann and Brobeck (3) found that very large doses of pitressin were the only means of producing a release of ACTH in rats with hypothalamic lesions.

This paper describes experiments that test, in the most direct fashion possible, the effect of various substances and tissues on the release of ACTH. Rat anterior pituitary tissue was incubated *in vitro* and the release of ACTH into the medium was measured by bioassay. The results indicate that the posterior pituitary probably plays a very important part in the release of ACTH after stress.

[1] *Manuscript received February 22, 1955.*
Contribution from the Allan Memorial Institute of Psychiatry, McGill University, Montreal, Que.

Method

Preparation of Tissues

Young adult (110–160 gm.) male rats of the Sprague–Dawley strain were anesthetized in the animal room by the intraperitoneal injection of nembutal sodium. Rats that were disturbed by the injection were not used. The sleeping rats were transported to the laboratory where the heads were removed with a guillotine. The top of the skull was removed with scissors, and the brain was carefully lifted out, exposing the hypophysis. The gland was dissected free with fine forceps, and the posterior lobe was lifted off and was placed into Krebs-Ringer-bicarbonate medium (12) containing 200 mgm.% glucose. The anterior lobe was then removed from the head, and the two tips of the lobe were cut off with fine scissors and were placed together into medium. Usually the anterior pituitary tissue of three rats was pooled in each experiment; one of the tips from each animal was used as the control tissue, and the remaining tips as the experimental tissue.

Hypothalamic tissue was dissected from the base of the brain and was cut into approximately 1 mm. cubes. The median eminence was included in the sample of hypothalamic tissue. Brain cortex and liver tissue cubes of similar size were also used.

Incubation

Incubations were carried out in Warburg flasks of 5 ml. capacity. The additions were usually as follows:

Medium		500 μl.
Anterior pituitary tissue	about	3 mgm.
dl-Epinephrine.HCl or *dl*-arterenol.HCl (4 mgm. per ml.)		10 μl.
Posterior pituitary tissue	about	2 mgm.
Brain cortex or hypothalamus	about	15 mgm.

The gas phase was 5% CO_2 – 95% O_2, temperature, 38°, and the incubation period, one hour. At the end of the incubation the medium was transferred to a small test tube containing 15 μl. of 1 *N* HCl, and the tissue into 100 μl. of 0.01 *N* HCl.

Analysis

For analysis of the media for ACTH, 15 μl. of *N* $NaHCO_3$ was added to neutralize the sample and 50 and 150 μl. aliquots were used as U_1 and U_2 in the *in vitro* bioassay of Saffran and Schally (7). The tissue was ground in the 100 μl. of 0.01 *N* HCl with a few grains of sand and a rounded rod. Aliquots of the extract were used for bioassay. The substances added to the pituitary tissue, for example arterenol, did not interfere with the bioassay of ACTH except when extensive oxidation occurred, as evidenced by excessive pigment formation; the formation of the pigment was minimized by acidification of the samples, and could be prevented entirely by the addition of ascorbic acid along with the arterenol or epinephrine.

Expression of the Results

The activity was expressed as the milliunits of ACTH released by 1 mgm. of anterior pituitary tissue in one hour. Most figures in this paper are accompanied by the 5% fiducial limits, calculated from the assay.

Results

ACTH Released by Unstimulated Anterior Pituitary Tissue

Rat anterior pituitary tissue released a mean of 5.4 milliunits (mU.) of ACTH per mgm. per hour. The release was not altered by the injection of 25 mgm. of cortisone acetate into the rat one hour prior to the removal of the anterior pituitary tissue (Table I). Table II shows the relative amounts of ACTH that are released and that remain in the tissue.

TABLE I

RELEASE OF ACTH FROM "UNSTIMULATED" ANTERIOR PITUITARY TISSUE

Treatment of rats	mU. ACTH released/mgm./hr.	5% fiducial limits of each assay, mU.
Nembutal anesthesia	6.1	3.8– 9.7
	5.7	4.2– 7.6
	6.0	2.9–13.1
	7.1	3.3–15.4
	4.2	0.8–22.3
I.P. saline one hour before nembutal anesthesia	4.9	3.5– 6.9
	3.7	2.0– 6.7
I.P. cortisone acetate one hour before nembutal anesthesia	5.2	4.1– 7.8
	5.4	3.7– 8.1
	5.3	2.3–12.0
Mean ± S.E.	5.36±0.313	

TABLE II

ACTH CONTENT AND RELEASE BY ANTERIOR PITUITARY

	Pituitary No.			
	1	2	3	4
Weight, mgm.	3.5	3.6	3.5	2.3
ACTH released in 1 hr., mU.	17	13	19	12
ACTH remaining after 1 hr., mU.	156	222	156	188
Total, mU.	173	235	175	200
ACTH released/mgm. in 1 hr.	4.9	3.7	5.4	5.3
Total ACTH/mgm.	49.4	65.3	50.1	87.0

Effect of Hypothalamus

Hypothalamic tissue, incubated with anterior pituitary tissue, had no detectable effect on the release of ACTH (Table III).

TABLE III

RELEASE OF ACTH IN THE PRESENCE OF HYPOTHALAMUS

Weight of hypothalamus, mgm.	ACTH released/mgm./hr., mU.	5% fiducial limits of each assay, mU.
12.0	5.3	2.9– 9.9
20.3	12.5	6.9–22.5
18.0	6.4	4.2– 9.7
23.0	7.2	3.0–17.2

Mean ± S.E. 7.85±1.60

Difference from mean unstimulated value (Table I) = 2.49

$t = 1.54$

$P > 0.1$

Effect of Sympathetic Amines

Neither epinephrine nor arterenol had any significant effect on the release of ACTH. Table IV illustrates the results with arterenol.

TABLE IV

EFFECT OF ARTERENOL ON ACTH RELEASE

ACTH released, mU.	5% fiducial limits of each assay, mU.
7.8	3.9–13.0
9.4	5.6–15.6
5.0	2.5–10.2

Mean ± S.E. 7.40±1.29.

Difference from mean unstimulated value = 2.04

$t = 1.945$

$P > 0.05$

Hypothalamus Plus Sympathetic Amine

When hypothalamic tissue plus a sympathetic amine were incubated with anterior pituitary tissue, the release of ACTH increased threefold (Table V). The increase was statistically significant.

TABLE V

RELEASE OF ACTH IN THE PRESENCE OF HYPOTHALAMUS PLUS EPINEPHRINE OR ARTERENOL

Amine added	Hypothalamic tissue, mgm.	ACTH released, mU.	5% fiducial limits, mU.
Epinephrine	25.7	17.8	15.3–21.3
	21.0	8.4	3.8–18.3
Arterenol	20.0	25.8	17.2–38.2
	23.6	12.2	8.0–18.4
	19.7	16.0	10.6–24.1
	18.5	14.5	6.1–34.4
	2.6 (Median eminence region only)	10.7	6.0–19.0
	7.4	15.8	8.3–30.3
		Mean ± S.E. 15.15±1.87	
Difference from mean unstimulated value = 9.79			
		t = 5.15	
		P = <0.001	

Brain Cortex Plus Arterenol

On the suggestion of Dr. M. M. Hoffman, brain cortex tissue was used as a control tissue to determine the specificity of the hypothalamus. Surprisingly, brain cortex plus arterenol stimulated the release of ACTH as well as hypothalamus plus sympathetic amine (Table VI).

TABLE VI

RELEASE OF ACTH IN THE PRESENCE OF BRAIN CORTEX AND ARTERENOL

Brain cortex, mgm.	ACTH released, mU.	5% fiducial limits, mU.
18.0	12.8	8.0–20.4
2.6	18.8	12.6–28.1
5.0	21.0	6.9–64.0
	Mean ± S.E. 17.53±2.45	
Difference from mean unstimulated value = 12.17		
	t = 4.93	
	P = <0.001	

Other Tissues and Neurohumors

Liver plus arterenol or hypothalamus plus either acetyl choline or serotonin were without great effect on the release of ACTH (Table VII). However, these are the results of single experiments and they cannot be taken as conclusive.

TABLE VII

EFFECT OF LIVER PLUS ARTERENOL, AND OF OTHER NEUROHUMORS PLUS NEURAL TISSUE ON THE RELEASE OF ACTH

Tissue	Weight, mgm.	Neurohumor	Concentration, millimolar	ACTH released, mU.
Liver	9.0	None	—	8.0 (5.2–12.1)
Liver	9.0	Arterenol	0.2	11.7 (7.4–18.4)
Brain cortex	23.3	Acetyl choline	0.2	10.4 (7.7–14.0)
Hypothalamus	24.4	Acetyl choline	0.2	11.4 (3.8–35.1)
Hypothalamus	22.2	Serotonin	0.2	10.9 (5.9–19.5)

Effect of the Posterior Pituitary

Posterior pituitary tissue alone did not stimulate the release of ACTH; however, posterior pituitary plus either arterenol, sphingosine, or hypothalamic tissue, caused the release of a large fraction of the ACTH in the anterior pituitary (Table VIII; cf. Table II). Dopamine (3,4-dihydroxyphenylethylamine), which resembles arterenol closely, stimulated ACTH release only feebly, if at all.

TABLE VIII

THE RELEASE OF ACTH IN THE PRESENCE OF THE POSTERIOR PITUITARY

Tissue added	Weight, mgm.	Amine added	ACTH released, mU.	5% fiducial limits, mU.
Posterior pituitary	1.3	None	11.3	(6.1– 21.2)
Posterior pituitary	2.0	None	7.8	(3.7– 16.5)
Posterior pituitary	4.2	Arterenol	37.2	(7.9–176.0)
Posterior pituitary	2.0			
plus		None	34.3	(5.4–219.0)
Hypothalamus	12.0			
Posterior pituitary	2.0			
plus		None	37.5	(19.8– 71.0)
Hypothalamus	11.0			
Posterior pituitary	2.5			
plus		Arterenol	41.5	(17.2– 99.5)
Hypothalamus	6.0			
Posterior pituitary	2.4	Sphingosine	28.7	(17.1– 48.0)
None		Sphingosine	10.4	(7.3– 14.7)
Posterior pituitary	1.1	Dopamine	14.6	(7.6– 28.1)

Discussion

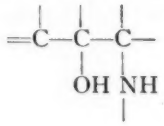
Role of Neurohypophysis

The experiments described in this paper point to an important role of the neurohypophysis in the pituitary–adrenal response to stress. The participation of neurohypophysial factors in the stress reaction was suggested by Rothballer (6) who observed a depletion of neurosecretory material in the hypothalamic–hypophysial tracts by noxious stimuli. Mirsky *et al.* (5) also suggested a relationship between the activity of the posterior pituitary and the release of ACTH. In addition, McCann and Brobeck (3) reported that a correlation exists between the polydypsia and the degree of the reduction in the response to stress in rats with hypothalamic lesions. Moreover, they found that only pitressin, in very large doses, reproduced the effects of stress in rats with effective lesions.

Role of Hypothalamus

Most of the evidence for the existence of a hypothalamic agent is indirect, and is based on the abolition of the response to stress in animals with discrete hypothalamic lesions (1). The only direct evidence for a hypothalamic agent is that of Slusher and Roberts (10) who prepared protein and lipid fractions of bovine posterior hypothalamus that caused eosinopenia and adrenal ascorbic acid depletion in intact rats. Until such extracts are tested in animals with effective hypothalamic lesions, their specificity and site of action are open to doubt. In our *in vitro* test, brain cortex was as potent as hypothalamus in releasing ACTH, when arterenol was present. Moreover, we found that the anterior and posterior parts of the hypothalamus were equally effective in discharging ACTH; this is contrary to the claims of Slusher and Roberts that extracts prepared from the anterior hypothalamus were devoid of activity.

The lipid extract prepared by Slusher and Roberts (10) would contain sphingosine, which would be derived from the sphingomyelin in the tissue. We found that sphingosine plus posterior pituitary tissue stimulated the release of ACTH from the anterior pituitary (Table VIII). It is of interest that sphingosine, arterenol, and epinephrine contain the following structure:



In dopamine, which is relatively inactive in our test system, the hydroxyl group is missing.

Role of Sympathetic Amines

Our finding that epinephrine or arterenol is needed with neural tissue for the stimulation of ACTH-release recalls Long's (2) evidence that suggested an important role for epinephrine in the pituitary–adrenal response to stress.

In the *in vitro* system, these amines may act as stimulators of the release of a factor that is present in or is formed by neural tissue, especially the neurohypophysis; moreover, these amines may also act synergistically with the brain factor directly on the anterior pituitary to release ACTH. The pathway of the pituitary–adrenal response to stress, incorporating these suggestions, is illustrated in Fig. 1.

We are now engaged in the isolation and characterization of the posterior pituitary factor.

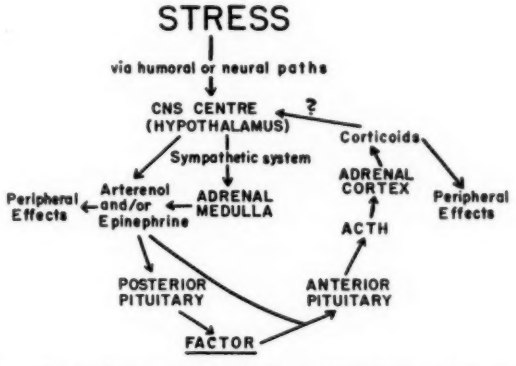


FIG. 1. The mechanism of the pituitary–adrenal response to stress.

Acknowledgments

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The sphingosine sulphate was supplied through the kindness of Dr. J. M. R. Beveridge, Queen's University, Kingston.

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QUANTITATIVE CHROMATOGRAPHIC METHODS FOR THE STUDY OF ENZYMIC TRANSPEPTIDATION REACTIONS[1]

By George E. Connell, Gordon H. Dixon, and Charles S. Hanes

Abstract

A colorimetric ninhydrin method has been applied to the quantitative estimation of amino acids and peptides on paper chromatograms. This method is suitable for the analysis of enzymic transpeptidation reactions. A procedure for the purification of filter paper for chromatography has been developed.

The study of enzymic transpeptidation reactions has suffered from the lack of a general method for their quantitative characterization. The requirements of such a method are exacting because of the complications which are often encountered in transpeptidation systems. A simple transpeptidation reaction might be represented as follows:

$$\text{A–B} + \text{C} \rightleftharpoons \text{A–C} + \text{B}. \qquad [1]$$

The transfer of the group A from the donor peptide A–B to the acceptor C results in the formation of the new peptide A–C, and the liberation of moiety B from the donor. If this reaction were proceeding in isolation, determinations of the change in amount of any one of the reactants or products would define its progress. In many of the transpeptidation systems studied to date the transfer reaction represented in Equation [1] is accompanied in varying degree by hydrolysis of the donor and the new peptide so that determinations of at least two of the chemical species involved are required to permit the progress of both hydrolysis and transfer to be followed. Frequently the analysis of the system is complicated further by the occurrence of additional transfer reactions, catalyzed by the same enzyme, which result in the formation of higher peptides, viz.

$$\text{A–B} + \text{A–B} \rightleftharpoons \text{A–A–B} + \text{B}\,, \qquad [2]$$

$$\text{A–B} + \text{A–C} \rightleftharpoons \text{A–A–C} + \text{B}\,. \qquad [3]$$

When such additional transfer reactions occur to an appreciable extent, the progress of any of the transformations in the system can be assessed reliably only when changes in all or nearly all the chemical species can be followed quantitatively.

Paper chromatography has been used extensively in the identification and qualitative study of transpeptidation reactions, and it has yielded much of the evidence for the widespread occurrence of the additional transfer reactions represented in Equations [2] and [3]. Accordingly the development of a rigorously quantitative method for the determination of amino acids and peptides separated on paper chromatograms has long seemed to us to offer the most promising general approach to the problem and we have directed our efforts to the development of such a method.

[1] *Manuscript received February 9, 1955.*
Contribution from the Department of Biochemistry, University of Toronto, Toronto, Canada.

Useful observations based upon other analytical techniques have been reported. A number of workers have applied isotopic methods to the problem (16, 10, 9, 23). Chromatography on resin columns was used by Dowmont and Fruton (6). Other studies have been made based upon group specific reactions, e.g. determinations of ammonia and of carboxyl groups liberated during the simultaneous hydrolysis of and transfer from amino acid amides (17); determination of hydroxamic acids formed by transfer of aminoacyl groups of hydroxylamine (16, 10, 23); determination of free arginine (18); liberation of cysteinyl-glycine and cysteine from glutathione (7, 14).

The importance of such results in indicating various features of transpeptidation reactions emphasizes the need for a more general and comprehensive system of analysis capable of application to a wider range of enzyme–substrate systems and, of equal importance, capable of yielding more detailed quantitative information about the complex of reactions which frequently occurs in any one system. The present paper is offered as a contribution toward this objective.

The Development of a Modified Ninhydrin Method

Our specific aim has been to develop a quantitative method for determining the amounts of amino acids and peptides in the individual spots as separated on filter paper chromatograms. High sensitivity and accuracy are required to permit measurement of the rates of the different reactions occurring in transpeptidation systems, and the method must have the capacity for numerous determinations. The application of the ninhydrin method to the quantitative chromatography of amino compounds has been studied by many investigators and on the basis of this considerable experience we have been able to evolve a procedure with the characteristics required for our purpose.

The least promising of the existing ninhydrin methods were those in which the color was developed on the paper and estimated either *in situ* by various means or else after elution. Even with the precautions described by Thompson and Steward (24) the color yield was found to be low and variable, the factors for different amino acids and peptides widely different, and the blank values high. As is evident from the literature, greater accuracy is possible with the more elaborate methods which rely upon detection of the spot on the chromatogram, excision of the spot and transfer of this portion of the chromatogram to a test tube, and development of the full ninhydrin color by a controlled treatment with a reagent of the type developed by Moore and Stein (20).

The first workers to adopt this approach (21, 1) encountered some difficulties, particularly due to high blank values and lack of reproducibility. These were overcome to some extent by Boissonnas (2, 3) and Fowden (8); both these authors found that high and variable blank values were due to the presence of ammonia in the paper and both devised alkali treatments to drive off ammonia prior to the determination.

One of the primary difficulties encountered in quantitative paper chromatography is the detection of the spots to be excised for analysis. In our experience the spots must be clearly outlined for accurate cutting. The "guide-strip" technique is unsatisfactory as a means of detection even on the best chromatograms.

The phenomenon of ultraviolet fluorescence of amino acids after heating on filter paper does not occur on purified filter paper and is probably due to impurities in unwashed paper reacting with the amino acid to form fluorescent material.

The preliminary spraying of the paper with a ninhydrin solution offered the possibility of the clearest and most detailed delineation of the spots, provided this treatment could be applied without decrease in final color yield. Appreciable losses in color were found to occur if such preliminary spraying, followed by heating at 65° C. to develop the color, were applied to chromatograms under neutral or alkaline conditions. But when the ninhydrin solution was appropriately acidified with acetic acid, no detectable losses in color occurred during the interval between detection of the spots and the subsequent full color development for analysis.

The methods previously reported for eliminating ammonia held by the paper were also modified. The methanolic-KOH solution of Boissonnas (3) was replaced by a less alkaline borate buffer in methanol to minimize the risk of color destruction at this stage. Instead of spraying the whole chromatogram prior to the detection of the spots, each spot was treated individually after excision, by flooding it with the borate buffer solution and then drying it in a stream of warm air. This procedure ensured complete elimination of ammonia and permitted rigid control of the amount of alkaline buffer added with each spot to the tube in which final quantitative development of color took place.

In the early stages of our work we adopted directly for the quantitative determinations the acetate-buffered ninhydrin reagent of Boissonnas (4) which is a modification of that of Moore and Stein (20). We found it desirable subsequently to modify the reagent by omitting the reducing agent (stannous chloride) and adding instead hydrindantin, a reduced form of ninhydrin. This change had the effect of reducing the blank values and improving the reproducibility of the determinations*. Later when a method was developed for preparing hydrindantin of higher purity these benefits were enhanced further. In connection with the quantitative determination a special tube was designed which facilitates greatly the complete development and extraction of the color.

** Moore and Stein have developed recently a modification of the ninhydrin reagent which is closely similar to our modification, and we are grateful to Drs. Moore and Stein for sending us a preliminary copy of their paper which is now in press in the Journal of Biological Chemistry. The new Moore and Stein reagent would seem to be better adapted to the analyses of amino acids and peptides in solution, being intended to be used with additions of 1 or 2 volumes of aqueous solution, whereas our reagent is intended for use without aqueous addition. We have included in Table II comparative values for the color yields obtained with a number of different ninhydrin reagents, including the new Moore and Stein reagent.*

As will be seen from Table II, the color yields for all but the few exceptional amino acids and for many peptides fall in a narrow range, i.e. 2.0–2.2, expressed as extinction coefficient at 570 mμ per micromole when measured in 10 ml. The values are slightly higher in general than those reported by Boissonnas and are closely similar to those reported recently by Troll and Cannan (25) who introduced a considerably modified reagent. It was hoped that this reagent might show some advantages for our purposes but trials showed it to be so weakly buffered as to be highly sensitive to the small amounts of acids and alkalies carried by the excised spots from some of our buffered chromatographic systems. It is possible that the Troll–Cannan reagent could be modified to overcome this difficulty.

Of importance to the application of the method to kinetic enzyme studies has been the development of a number of simple devices which facilitate the carrying out of relatively large numbers of determinations in batches up to 18 in number.

Chromatography

Descending one-dimensional chromatography has been used, usually with propanol–water (80/20 v/v) as solvent and with a period of at least two hours equilibration before addition of solvent to the trough (with magnetic 'fanning' inside the jar). In these respects the procedures have been similar to those described in earlier studies on transpeptidation reactions by Hanes, Hird, and Isherwood (11, 12). Valuable innovations have been (*a*) more effective purification of the paper, (*b*) for certain purposes impregnation of the paper with selected buffers and salts either to effect particular separations or to overcome salt effects, and (*c*) the use of elevated temperatures. The latter two developments will be discussed elsewhere in connection with their particular applications but the washing of the chromatographic paper requires general comment here.

Purification of the filter paper results in marked improvements in the chromatography of amino acids and peptides in nearly all the systems tried. The advantages include generally the formation of more compact and circular spots (including elimination or reduction of the 'forward trailing' which usually occurs with aromatic amino acids), freer and more rapid movement, and cleaner separations. These improvements are of such importance in quantitative chromatography as to make rigorous purification of the paper highly desirable. This is equally true when substances are to be taken off the paper for further examination. The procedure to be described below yields a more satisfactory paper than we have been able to obtain by any other procedure (cf. methods described earlier (12, 13, 15)).

Procedure for Washing the Filter Papers

Whatman No. 3 paper, mold-made in sheets about 21½ × 29 in., has been used. (Note—This is not No. 3 MM, which we have not tried.) The paper is cut lengthwise into two by guillotine and the half sheets about 10¾ × 29 in.

are washed in blocks of 60 sheets in a continuous process occupying in all about 52 days. The washing requires little daily attention and four blocks of papers (240 sheets in all) are treated simultaneously on a single rack. This capacity could be increased with little extra labor.

The essential parts of the washing equipment shown in Fig. 1 are (*a*) a wooden stand carrying a hinged supporting board (to which the blocks of paper are clamped) which can be locked in four positions; (*b*) a trough of wood with a polyethylene liner ($3\frac{1}{2} \times 5 \times 12$ in.) into which the papers dip and from which the extracting liquids rise by capillarity; (*c*) a 'suction head', of perspex, which is clamped across the top of each block of paper and through which the extractant is sucked after it has risen up through the paper; by this removal of extractant at the top of the block of paper, and its replacement by air, capillary suction capacity is regenerated so that the upward flow

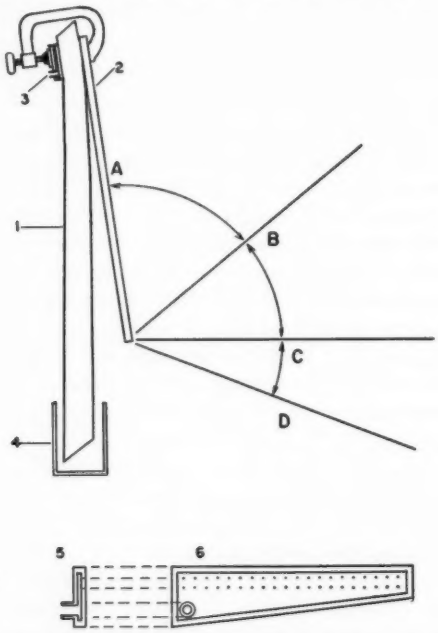

FIG. 1. Diagram of the assembly for washing the chromatographic paper.

The blocks of 60 sheets of filter paper (1) are clamped to the supporting board (2) which is hinged along its bottom edge. This board can be swung to any of the four positions shown at *A*, *B*, *C*, or *D* and locked in any one of these positions. As shown in Fig. 2, the supporting board is locked in position *A* and the filter paper is hanging freely downwards. This position is used only for the initial wetting of the paper by capillarity (to eliminate wrinkling); the bulk of the washing treatment thereafter is carried out with the board, and the filter paper blocks, in the inclined position *B*. The suction head (3) is held firmly against the top of the block of paper by means of a pair of G-clamps, which serve also to hold the block in place on the supporting board. Suction is applied via the tubulature shown at (3) and the effluent liquid is led to a collector bottle. Further details of the suction head, constructed of perspex plastic, are shown in (5) and (6). The ends of the papers forming the block dip into the extracting liquid contained in the trough (4) which is supported on appropriate blocks.

of extractant drawn by capillarity continues—at a rate of about 1.5–2 liters per 24 hr.—so long as suction is applied to the 'head'; (*d*) a collector system comprising a large collecting bottle, evacuated by filter pump, and individual small collectors permitting inspection or analysis of the effluent from each block of paper; (*e*) two large polyethylene bags in which each block of paper and its trough is totally enclosed throughout the treatment, the suction head being clamped over a 'window' cut in this covering and the extractants being added to the trough through a slit closed normally by pressure tape.

The above description applies to an installation in which each block of paper is treated as a unit, being provided with its own trough and being encased in its own pair of polyethylene bags. For the steady operation of the washing process on a larger scale, we have found it more convenient to provide a single long trough into which all the papers dip (e.g. a trough 52 inches long will serve for the simultaneous treatment of four blocks of paper); this trough is constructed of plywood and lined with a folded polyethylene sheet 0.004 inches thick. In this modified arrangement the individual wrapping of the blocks is omitted; instead the blocks and the trough are encased in a single large sheet of polyethylene.

The washing process includes the sequence of treatments indicated in Table I.

TABLE I

Treatment	Extractant and volume, in liters, used per block of 60 sheets		Duration of extraction, days	Suction
1	2 *N* acetic acid,	4–5	3	No suction
2	Water,	7	4	Suction continuous
3	0.5 *N* lithium hydroxide,	12	22	Suction intermittent
4	Water,	10	9	Suction intermittent at first, then continuous
5	0.1% (w/v) calcium acetate,	4.5	3	Suction intermittent
6	Water,	8	5	Suction continuous
7	95% (v/v) ethanol,	6	4	Suction continuous

With the supporting board in position *C* (Fig. 1) the piles of dry paper are placed in position, each in its polyethylene bag being clamped by two G-clamps applied to the suction head (protected by a wooden batten). The supporting board is then turned up to position *A* so that the papers hang vertically. The trough is now supported in position and the first extractant (2 *N* acetic acid) is run into it. As this rises in the paper by capillarity more is added. The paper is allowed to hang vertically during this preliminary wetting to eliminate the wrinkling which otherwise occurs due to expansion. Once the solution has reached the top the board is turned to position *B* where it will remain for all but the seventh treatment. The block is allowed to imbibe the acetic acid for three days and then any excess is removed from the trough (through a suction tube).

Suction is now applied and the effluent, pale yellow at first, is drawn from the suction head. Water is placed in the trough and treatment 2 is begun. By the end of four days the bulk of the acid is removed, the effluent containing now about 0.1 *N* acid which is low enough to commence treatment 3.

The extraction with 0.5 *N* lithium hydroxide, treatment 3, is the most important one in the process. Under the influence of lithium hydroxide the structure of the paper fibers appears to be opened up more effectively than with any other alkali we have tried and much non-cellulosic material, colored and otherwise, comes slowly into solution. The 'break through' of the lithium hydroxide front occurs about two to three days after this extractant is placed in the trough and is evident from the brown color of the effluent. Thereafter only intermittent suction is applied, e.g. five to six hours daily for the first week and five to six hours every two to four days subsequently. The purpose of this is twofold, (*a*) to prevent the drying out and 'caking' of the block in the region of the suction head, and (*b*) to conserve the lithium hydroxide. It was found that once the first flush of impurities is removed by this extractant, the rate of further removal is slow, governed apparently by the rate of diffusion from the inside to the outside of the fibers so that the maintenance of a continuous flow of extractant is unnecessary, diffusion proceeding during the intervals between the periods of suction. By the end of the prescribed treatment, the effluent obtained after a three-day period without suction shows no visible color when viewed in 15 cm. depth in a tube. (It still contains appreciable amounts of dissolved material including substances absorbing strongly in ultraviolet light, absorption maximum at 265 mμ. The complete disappearance of this material from the effluent requires about 40 days of extraction but this is unnecessary for any use to date.)

During treatment 4 the suction is intermittent for the first three days but continuous thereafter. By the end of the prescribed treatment the effluent should be less than 0.01 *N* in alkali.

Treatment 5 (and the consequent treatment 6) is a requirement peculiar to the chromatography of amino acids and peptides so far as we are aware. Unless a divalent cation, of which the paper was stripped in treatment 1, is replaced, the chromatograms show serious streaking. Treatment 6 serves to remove all but the traces of calcium ion bound to the paper.

Treatment 7 is carried out with the board first in position *C* with suction applied until the alcohol front 'breaks through' and then in position *D* without suction, when the alcohol merely siphons through the block and is allowed to drain out through a hole cut in the polyethylene bag. The purpose of this final washing is twofold, (*a*) to remove remaining impurities including a small amount of yellow material and a fluorescence visible under ultraviolet light, and (*b*) to assist in the final drying of the paper. This latter is conveniently done after removing the suction head by perforating the blocks of paper with a cork borer so that the papers can be suspended individually, with spaces between, on two parallel rods.

Details of the Analytical Method

Special Chemicals

Ninhydrin.—This was purchased from Dougherty Chemical Co.

Hydrindantin.—This was prepared at first according to the method of Ruhemann (22) and then by that of MacFadyen and Fowler (19). Subsequently the following modification was adopted: Ninhydrin (10 gm.) is dissolved in 250 ml. 0.5 *N* acetic acid at 30°. Ascorbic acid (5 gm.) is added and dissolved. The solution is warmed to 65° with stirring when a copious crop of white crystals of hydrindantin begins to appear. After 10 min. at 65° further ascorbic acid (2 gm.) is added and the temperature maintained for an additional five minutes. The suspension is then cooled rapidly. The hydrindantin is filtered off on a sintered glass funnel, washed twice with distilled water, and dried *in vacuo* over sulphuric acid. The yield is about 7.5 gm. and the product is used without recrystallization.

Glacial acetic acid.—The best grade acid was treated with chromic anhydride and twice redistilled to remove traces of ammonia.

Ethylene glycol monomethyl ether.—Methyl "Cellosolve" (as purchased from Carbide and Carbon Chemicals Co.) was redistilled from ferrous sulphate to remove all traces of peroxides which cause rapid fading of Ruhemann's purple. The purified solvent was held at −20° until used.

Lithium hydroxide.—This was purchased from British Drug Houses.

Preparation of Solutions

Detection spray.—Ninhydrin (50 mgm.) was dissolved in 75 ml. absolute ethanol plus 25 ml. 2 *N* acetic acid. For certain alkaline paper chromatograms (for example lithium carbonate impregnated paper) 10 *N* acetic acid was substituted for the 2 *N* acid.

Borate buffer in methanol.—Boric acid, 9.28 gm., was dissolved in methanol and 32.5 ml. 6 *N* potassium hydroxide solution added and the solution made up to 500 ml. When this buffer is allowed to evaporate and is redissolved in an equal volume of water, the resulting pH is approximately 11.5.

Quantitative ninhydrin–hydrindantin reagent.—Ninhydrin (380 mgm.) and hydrindantin (120 mgm.) were dissolved in 50 ml. methyl 'cellosolve' plus 50 ml. *N* acetic acid – sodium acetate buffer, at pH 4.63. This buffer solution is conveniently prepared by the method of Cole (5) as follows: 500 ml. *N* NaOH solution is neutralized with glacial acetic acid from a burette using phenolphthalein as indicator; an additional volume of acetic acid equal to that required for neutralization is then added and the volume is brought to 1 liter. The reagent is normally prepared in large batches, 1–4 liters, and is held at 0° under nitrogen in a special dispenser bottle.

Eluting solvent.—50% (v/v) aqueous ethanol.

Procedure

Cleanliness of glassware, benches, etc. is essential and no part of the chromatogram to be analyzed must be handled; large flat polyethylene bags have proved invaluable for temporary storage of chromatograms.

The chromatogram is air dried and the detection spray is applied with an all-glass atomizer at the rate of about 1 ml. per 150 cm.^{2} of paper. (This refers to Whatman No. 3 hand-made or mold-made paper.) The color is then developed by heating to 65° for 5–10 min. A photographic print is usually made for record at this stage, using light transmitted through the chromatogram acting on copying photographic paper, for example Kodagraph paper.

The spots for analysis are delineated in pencil using celluloid templates of appropriate size and shape; these range from 3 cm.^{2} to 12 cm.^{2} in area. Areas of paper of the same size are marked out for 'blank' determination from the same chromatogram. The areas are cut out and, held in forceps, each is flooded with methanolic borate buffer, delivered from a semiautomatic pipette of 0.25–0.30 ml. capacity (Fig. 2 *A*). This volume is sufficient to wet the whole of the largest size of paper; if one of the smaller pieces will not hold the full volume, the excess is added to the tube in which the final color development of this spot will later take place. (If it should be desired to treat an area of paper greater than the largest mentioned, two deliveries of half-strength methanolic buffer may be used.)

The wetted spots are then suspended by stainless steel clips from small racks (Fig. 2 *B*) and these are placed for five minutes in a miniature tunnel dryer through which a current of warm air (40°) is passed (from a domestic hair dryer).

Each piece of paper is now rolled up (using forceps and polyvinyl 'trough'—see Fig. 2 *C*) and placed in the bottom of a reaction tube (Fig. 2 *D*). The quantitative ninhydrin–hydrindantin reagent (2 ml.) is now added from a

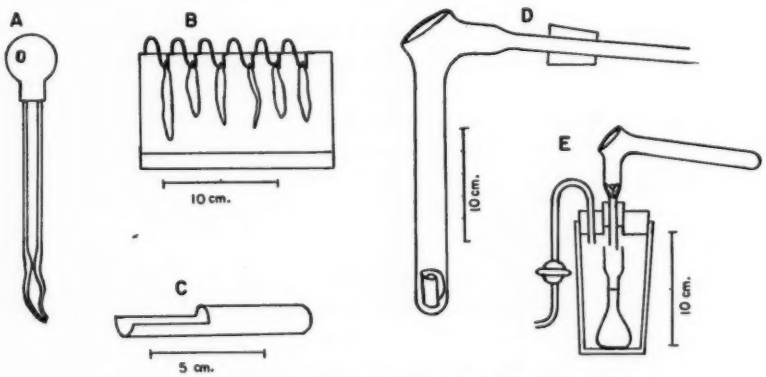

FIG. 2. Items of apparatus designed to facilitate the analyses.
A. Semiautomatic pipette for the methanolic borate buffer.
B. Rack of stainless steel, with clips to hold six 'spots' excised from chromatograms.
C. 'Trough' cut from polyvinyl tubing.
D. Tube designed for the development of the ninhydrin color and for its subsequent elution from the filter paper 'spot'.
E. Simple assembly showing position of the tube for elution of the color.

simple automatic pipette. The reaction tubes, in a copper rack which will take 18 tubes, are now placed in a boiling water bath for 20 min. and are then cooled in running water.

The same reaction tube is now used for the quantitative elution of the Ruhemann's purple from the paper. The tube is turned over and its 'funnel end' inserted into a stoppered jam jar which can be evacuated. The piece of paper is transferred to the throat of the funnel with a pointed glass rod, preferably with all cut edges directed upwards, and it is tamped down lightly (Fig. 2 *E*). The reagent, followed by successive washings with 50% ethanol, is now sucked through the paper pad into the volumetric flask (10 or 25 ml.) inside the jar; the 10 ml. flask is used for quantities of amino acids up to about 0.25 μM. and the 25 ml. flasks for larger amounts. The extracts, after being made to volume, are thoroughly mixed and the extinction coefficients at 570 mμ are then determined, preferably within one hour. For this purpose we have used a Beckmann Model B spectrophotometer with cells of 1 cm. path length.

Standardization of the Ninhydrin Method

The color yields given by various amino acids and peptides have been determined at different levels both with and without development on chromatograms. For the standardization of the ninhydrin method *per se*, known volumes of standard solutions of the amino acids and peptides were delivered by micropipette on to squares of the standard filter paper. After drying, these were analyzed in the way described above, blank paper squares being analyzed at the same time. In Table II are given results obtained by this method expressed as extinction values at 570 mμ (E_{570}) per micromole of amino acid or peptide, the absorption being measured in 10 ml. or calculated to this volume when measured in 25 ml. For comparison, the corresponding values reported by Boissonnas (4) and by Troll and Cannan (25) are included. In addition values for a recently devised reagent of Moore and Stein (unpublished; see footnote to p. 418) are given in Column 5.

An analysis of the data upon which the values given by Column 2 of Table II have been based permits a definition of the accuracy of the actual analytical procedure. For 28 sets of readings in triplicate, covering the range 0.3–0.6 μM. of amino acid or peptide, the standard deviation of an individual reading was $\pm 0.4\%$; to this should be added an almost negligible error introduced in subtracting the blank values.

These errors pertain to the analytical procedure only and indicate the degree of reliability of the processes of development, elution, and determination of the color. Additional errors are introduced in the routine application of the method to the analysis of the spots excised from chromatograms. An equally precise general statement on the magnitude of such errors is not possible since a major factor is the perfection of the chromatographic separation of the amino acid or peptide in question. We have found that standardization factors for a number of amino acids and peptides as determined on spots

TABLE II

EXTINCTION COEFFICIENTS AT WAVE LENGTH 570 mμ FOR DIFFERENT AMINO COMPOUNDS

	E_{570} values per micromole in 10 ml.			
(1) Compound	(2) Present method	(3) Boissonnas (4)	(4) Troll and Cannan (25)	(5) Moore and Stein*
Alanine	2.17	2.06	2.20	1.99
Arginine	2.14	2.21	2.11	2.07
Asparagine	1.51	—	—	1.95
Aspartic acid	2.02	1.89	2.13	1.93
Cysteine (maleimide)	(1.34)	—	—	—
Glutamic acid	2.20	1.90	2.14	2.03
Glycine	2.11	2.04	2.11	1.95
Histidine	2.13	1.93	2.20	2.09
Leucine	2.09	2.04	2.18	2.05
Lysine	2.40	2.22	2.38	—
Methionine	2.18	2.04	2.20	2.09
Phenylalanine	2.02	1.92	2.18	2.05
Serine	2.02	1.94	2.14	1.95
Threonine	2.09	1.91	2.21	—
Tyrosine	1.92	1.83	—	2.05
Valine	2.13	2.03	2.16	1.99
γ-Glutamylglutamic acid	2.07	—	—	—
γ-Glutamylglycine	2.44	—	—	—
γ-Glutamylphenylalanine	2.02	—	—	—
Glycylglycine	2.06	—	—	—
Glycylleucine	2.04	—	—	—
Glycylphenylalanine	1.94	—	—	—
Glycyltyrosine	2.05	—	—	—
Glycylvaline	1.97	—	—	—
Leucylglycine	2.21	—	2.06	—
Glycylglycylglycine	1.73	—	—	—
Glycylglycylleucine	—	—	1.93	—
Glutathione (maleimide)	(2.10)	—	—	—
Glutathione—oxidized	2.06	—	—	—
Ammonia	2.01	—	0.63	—

** These values are derived from those reported for the new reagent of Moore and Stein to which reference has been made in the footnote to p. 418. The original values were given, however, relative to leucine (= 100) but it is stated that leucine gave 95% of the theoretical value and this is known to be 2.17 (25); the values in Column 5 have been derived on this basis.*

excised from run chromatograms agree to within two per cent of the values given in Table II (Column 2).

The magnitude of the blank paper value deserves separate mention; the blank values with areas of filter paper ranging from 3–9 cm.² are found to fall in the region of 0.04–0.05 (E_{570} values). Most of the color contributing to this blank value is due to the reagent itself, and the area of the paper on which the determination is made affects the value only slightly, the E_{570} value increasing by about 0.002 per cm.² of paper. It should be mentioned that the paper blank value has been found to be increased by tobacco smoke, and reproducibility of results is improved when there is no smoking in the laboratory.

The method has been applied during the past two years to the study of the kinetics of various transpeptidation reactions and we find that the values can be relied upon to within 0.01 of a micromole of amino acid or peptide.

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EXERCISE AND TEMPERATURE REGULATION IN LEMMINGS AND RABBITS[1]

By J. S. Hart and O. Heroux

Abstract

Oxygen consumption and body temperatures were determined in lemmings at environmental temperatures from 20° C. to −10° C. and in rabbits from 20° C. to −50° C. Body insulation indices were estimated as the ratio $\frac{\text{body temperature minus air temperature}}{\text{oxygen consumption}}$. In both species, increase in activity and decrease in temperature led to increases in oxygen consumption that were additive over the temperature range. Oxygen increments of work were independent of environmental temperature in the absence of progressive hypothermia. Work led to increases in body temperature at the upper environmental temperatures and to decreases in body temperature at the lower temperatures. In extreme cold, rabbits became progressively hypothermic during work and there was a decline in oxygen consumption. Body temperatures started to fall at environmental temperatures 18° C. higher in working than in resting rabbits. Insulation was lower in working than in resting animals. During exercise there appears to be a readjustment of body temperature, insulation, and heat loss until thermal equilibrium is established. The regulation of heat production, within limits, seems to be independent of body-temperature changes during exercise.

Introduction

Previous studies on mice (2, 3) have shown that heat produced by forced work (running) was dissipated and therefore not available for thermoregulation in cold environments. The increased dissipation of heat during work was associated with decrease in body insulation, and at low temperatures, with a fall in body temperature. Since the behavior of mice might be different from that of larger or better insulated animals, further tests with lemmings and rabbits have been carried out to determine the effect of exercise on metabolism, body temperature, and insulation at various environmental temperatures.

Methods

Oxygen consumption and colonic temperature were determined during rest (minimal activity (3)) and work (running) at various environmental temperatures. From these data, an index of body insulation, $\frac{\text{body temperature minus air temperature}}{\text{oxygen consumption}}$, was derived for comparing the same group of animals under different conditions.

All animals, prior to testing, were trained to run under the conditions of the tests and were acclimated for several weeks at 20° ± 1°C. Between tests they were kept at 20° ± 1° C. and received mixed grain, fresh vegetables, and water ad libitum.

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Lemmings

Eight* adult lemmings, *Dicrostonyx groenlandicus groenlandicus* (average weight 60.9 gm., range 45 to 77 gm.) from the Fort Churchill, Man., area were tested at various times of the year. Body temperatures were measured after 1½ hr. exposure at temperatures of −10°, 0°, 10°, and 20° (±2° C.) by inserting thermocouples 3 cm. into the colon. Temperature recording, which was done after light ether anesthesia, required 30 to 45 sec. Immediately after recovery from anesthesia, each animal ran for 20 min.† in the activity wheel (2) at 15 cm. per sec. without stimulation and then body temperatures were again recorded after anesthesia.

Oxygen consumption was measured at different times at the same exposure temperature used for body temperatures in a closed circuit system with an activity wheel – metabolism apparatus (2). After a thermal equilibration period of 1 to 1½ hr., oxygen consumption was recorded for one hour during rest. The data obtained during intermittent periods of excessive visible activity were excluded from the average. Oxygen consumption was then recorded for 15 min. during running at 15 cm. per sec. after a thermal equilibration period of 10–15 min. running.

Environmental temperatures in the activity apparatus recorded by thermocouples within the chamber were 1° to 2° C. higher than the equivalent temperatures used for body temperature tests.

Rabbits

Each of six littermate rabbits (three males, three females; average weight 2.54 kgm., range 2.00–3.02 kgm.) was tested at six temperatures from +20 to −50° C. chosen in random order to minimize the effect of progressive change in uncontrolled factors during the course of the tests. The rabbit was first placed on a canvas treadmill (Fig. 1) at the desired temperature for 40 to 50 min. and held in place by a bottomless cage. The windowed lid section of the apparatus was then lowered into the mineral oil moat on the lower section, a circulating fan mounted inside the lid was switched on, and a dried air stream was withdrawn at the rate of about 100 ml. per min. from the chamber through a Beckman oxygen analyzer (model C). Entrance air was via small holes in the chamber. Twenty minutes after closure of the metabolism apparatus, readings on the oxygen analyzer were taken every 5 min. for 40 min. and mean oxygen consumption rate was calculated from the fall in chamber oxygen pressure in the time interval $(t_1 - t_2)$ according to the formula:

$$\frac{\Delta P_{O_2}{}^{t_1 - t_2}}{760}\left(\frac{\text{chamber vol.} \times 273}{\text{chamber temp. (°K.)}} + \frac{\text{vol. withdrawn from chamber} \times 273}{\text{temp. (° K.)}}\right).\ddagger$$

The apparatus was then opened and the rabbit fastened to a holding board at test temperature while colonic temperature was recorded by thermocouples

** Tests actually initiated on 16 animals, but eight died during the tests.*

† Preliminary tests indicated that body temperature change during exercise was complete in approximately 20 min.

‡ This formula does correct for small errors resulting from: (1) differences in volume of air entering and leaving chamber, and (2) differences in % O_2 in air entering and leaving chamber.

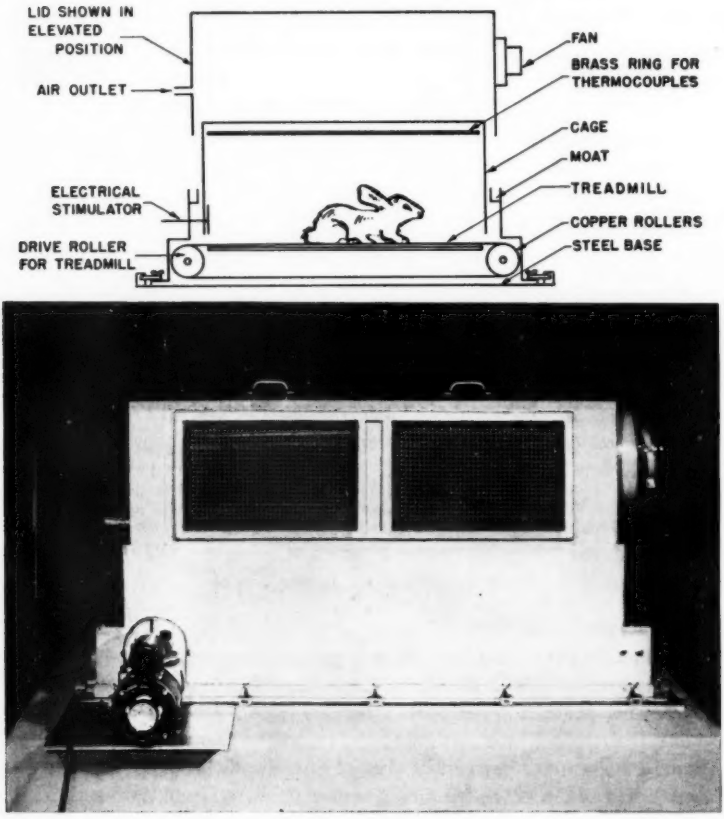

FIG. 1. Rabbit metabolism apparatus.

inserted to a depth of 6.5 cm. Body temperature determinations, taken without anesthesia, required about five minutes.

Immediately afterwards, the rabbit was replaced in the chamber and oxygen consumption was measured for 20 min. during running at 1.3 m.p.h. after an equilibration period of 10 min. running. Running was promoted by occasional electrical stimulation. Body temperatures were again recorded immediately after cessation of running.

Environmental temperatures in the activity apparatus were recorded by thermocouples soldered to the chamber cage and to a brass ring projecting into the cage.

Results

Lemmings

Heavy mortality was experienced in tests with lemmings; 8 out of 16 animals died, four while in holding cages at 20° C. and four while running at

20° C. or higher. Of the latter, one died at 28° C. after his oxygen consumption increased from an initial value of 190 to over 500 ml. per hr., one died at 25° C. after running for five minutes, and two died at 20° C. with body temperatures of 41° and 42° C. These animals all appeared to suffer heat stroke with abrupt cessation of activity, gasping respiration, and loss of voluntary muscular control. The upper environmental temperature limit appears to be around 20° C. during running, but higher temperatures could be tolerated at least temporarily if the lemming remained at rest.

The average values for the eight lemmings that survived all tests show that work produced approximately the same increase in oxygen consumption at all temperatures (Fig. 2).

Body temperatures (Fig. 2) were relatively constant during rest. After work, however, mean body temperatures were elevated above the resting level at temperatures above 0° C. but were depressed below the resting level at −10° C. Although the variability was such that the ranges overlapped at −10° C., the body temperature of each animal was lower when at work than when at rest. Similarly, at temperatures above 0° C., the body temperature of each animal was higher when it was working than when it was at rest.

Body insulation indices of lemmings during rest and work at various environmental temperatures (Fig. 2) increased with fall in environmental temperature and, at all temperatures, work produced a fall in body insulation.

Rabbits

Rabbits, like lemmings, showed large individual variations in their reactions to temperature and work. The body temperature of each animal decreased during work at the lowest temperatures and this hypothermia was associated with lowered willingness to run. Two rabbits with the largest fall in temperature did not run for more than ten minutes at −50° C. and one of them ran with difficulty at higher temperature.

The average oxygen consumption and body temperature of the six rabbits during rest and work at different environmental temperatures are shown in Fig. 2. Statistical examination of the data indicated that the increase in oxygen consumption with decreasing environmental temperature was significantly less during work than during rest. At temperatures above −20° C., work caused increases in oxygen consumption averaging 3.4 liters/hr. and increases in body temperature ranging from 0.3° C. at −20° C., to 1.6° C. at +20° C. Below −20° C., however, oxygen increments of work declined and there was a progressive fall in body temperature. All rabbits maintained body temperature more effectively during rest than during work at temperatures below −20° C.

As noted in lemmings, work in rabbits was associated with lowered insulation index at each environmental temperature (Fig. 2). At both levels of activity there also was a rise of insulation with fall in environmental temperature. During rest, insulation appeared to be relatively constant between −10° and −35° C.

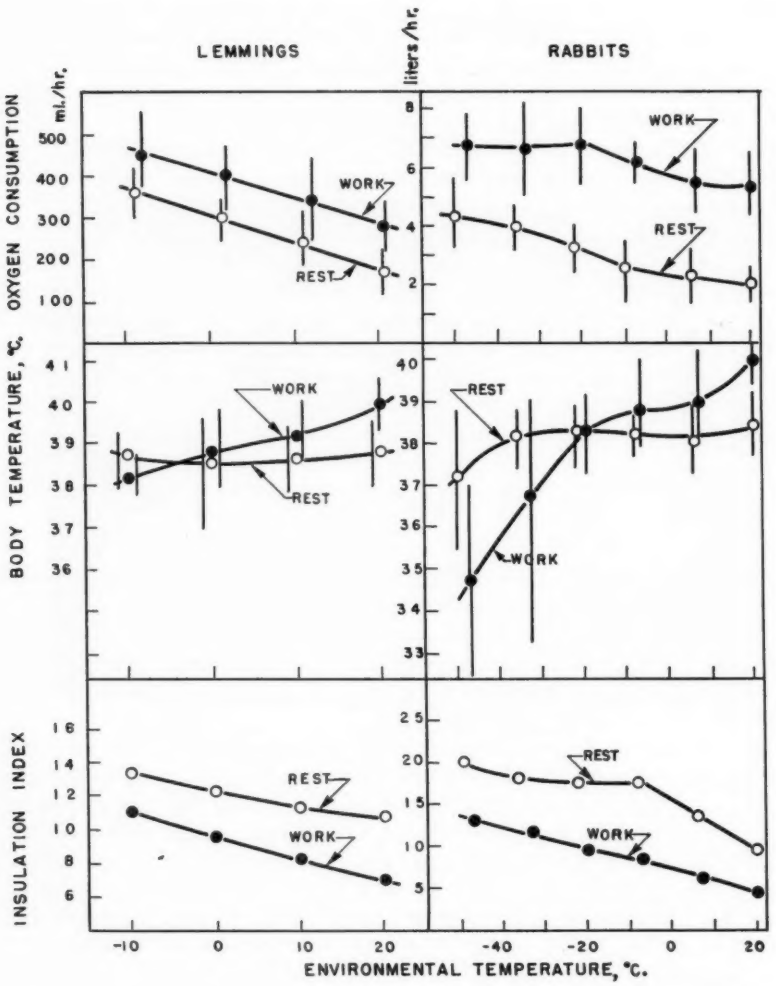

FIG. 2. Mean oxygen consumption, body temperature, and insulation of lemmings and rabbits during rest and work at different temperatures. Range of variation given by vertical bars. Data on only four of the six rabbits obtained during work at lowest test temperature.

Discussion

The results of this study, which confirm previous findings on mice (3) and rats (1), indicate that exercise in cold environments can have a deleterious effect on temperature regulation. This tendency was marked in rabbits and would probably have been also observed in lemmings if lower temperatures had been used. On the average, body temperatures of working rabbits started to fall (below the 38° C. resting level) at environmental temperatures approximately 18° C. higher than those of resting animals.

In mice, lemmings, and rabbits, work produced an increase in body temperature at high environmental temperatures and a decrease at low environmental temperature. At an intermediate point henceforth designated as the "crossover" temperature, exercise had no effect on body temperature. In mice, lemmings, and rabbits acclimated to 20° C., the crossover temperatures are estimated to occur at about 13° C. (3), −4° C., and −20° C. respectively. The species differences are undoubtedly related to differences in body size and insulation.

Unless there was a marked fall in body temperature, work produced approximately equal increments in oxygen consumption at all environmental temperatures, indicating that the metabolic effect of work was added directly to that of cold. In rabbits, however, the observed fall in oxygen increments at the lower test temperature might suggest that the heat produced by the exercise had partially substituted for the extra heat produced in response to cold. While this is possible, it is quite improbable because the magnitude of the hypothermia of the rabbits working at such temperatures shows that they did not produce sufficient heat for maintenance of body temperature.

Insulation in lemmings and rabbits decreased with exercise, and increased with fall in temperature under both rest and exercise conditions. Therefore, unlike the majority of species studied by Scholander (5), these species did not obey Newton's law of cooling (4) even during rest. However, various factors such as body temperature changes and uncertain attainment of thermal equilibrium make interpretation of insulation changes difficult in these species. Intermittent activity of lemmings during the rest period was another complicating factor.

The observed body temperature changes from rest to the end of exercise provide some information on readjustments in thermal balance throughout the exercise period. From these temperature changes, it can be concluded that, during exercise, heat production exceeded heat loss above the crossover temperature and was less than heat loss at lower temperatures. Therefore, as shown schematically in Fig. 3, heat loss during exercise increased much more than heat production with fall in environmental temperature. As exercise continues, however, it is postulated that the animals tend to reach a new thermal balance with the following readjustments. Above the crossover point, the rise in body temperature caused by the excess of heat production over heat loss increases the gradient from body to air. This in turn causes an increased heat loss. Thermal balance is attained when heat loss rises

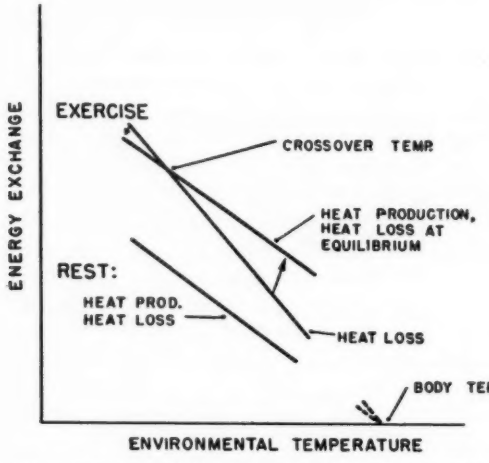

FIG. 3. Temperature regulation during exercise. Explanation in text.

until it equals heat production and body temperature remains steady at a new higher level. Below the crossover point, the opposite changes are postulated. The excess of heat loss over heat production lowers body temperature, which in turn reduces the heat loss. Balance is attained when heat loss falls until it equals heat production and body temperature falls to a new equilibrium level. The suggested changes in heat loss are shown by arrows in Fig. 3.

Since only initial and final body temperatures were obtained, the extent to which thermal equilibrium was attained during exercise cannot be ascertained. However, from previous results on mice (3) it is known that equilibrium was reached after 15–20 min. running. Preliminary tests on lemmings in which body temperatures were measured at intervals throughout exercise suggested that thermal balance was reached in 20–30 min. It therefore seems probable that the body temperatures obtained after exercise were approaching equilibrium. Because of their greater size rabbits would reach stable temperatures much more slowly than lemmings. Greater size and larger body temperature changes indicate that thermal balance was probably not fully attained in rabbits. Whether or not equilibrium was fully reached, it is believed that changes in heat loss similar in direction to those depicted in Fig. 3 occur during exercise in the species studied.

The studies in mice, lemmings, and rabbits raise a number of problems in temperature regulation, the most important of which concerns the apparent failure of the heat produced by work to substitute for the heat produced during exposure to cold. Furthermore, it is puzzling that during exercise the extra heat elicited by cold is similar in magnitude to that produced at rest in spite of the differences in body temperature during the two states, i.e. the regulation of heat production seems to be *independent of body temperature changes during exercise.* Finally, the factors that determine the crossover temperature

remain to be explored. From earlier studies on cold- and warm-acclimated mice (3), it is apparent that the crossover temperature reflects the ability of the animal to offset the fall in body insulation caused by exercise. The cardiovascular adjustments that must be involved will be the subject of further research.

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A STANDARDIZED METHOD FOR THE PRODUCTION OF HEMORRHAGIC SHOCK IN THE RAT[1]

By H. G. Downie[2] and J. A. F. Stevenson

Abstract

Although the blood pressure is one of the important criteria in the standardization of hemorrhagic shock in the dog, it has rarely been used for this purpose in the rat. A method resembling the reservoir technique developed by Wiggers and Werle (1942) for the dog using blood pressure as the criterion has been modified for use with the rat. Male Sprague-Dawley rats weighing approximately 400 gm. were used. In the standardization of this technique the blood pressure was reduced to 30 mm. Hg in a 10-min. period of hemorrhage and then maintained at this level by subsequent small hemorrhages into the reservoir until reinfusion indicated the beginning of vascular collapse, at which time all the blood in the reservoir was returned. Considering that those animals which lived longer than 48 hr. were survivors, in a series of 27 animals, 21 died and 6 survived—a mortality rate of 78%.

During the hypotensive period there was a consistent and steady drop in the respiratory rate and rectal temperature. The heart rate declined initially and tended to recover as the hypotensive period progressed. After reinfusion the blood pressure rose but did not reach prehemorrhage levels. Hemorrhage into the bowel and convulsions were significant postreinfusion findings.

Although the blood pressure is one of the most important criteria used in the study of standardized hemorrhagic shock in the dog, it has rarely been used for this purpose in the rat. In nearly all of the important investigations on the metabolic effects of hemorrhagic shock which have been carried out in the rat, the standardization of the procedure has depended upon some estimate of the blood lost, either expressed as a per cent of body weight (3, 4, 5, 10) or surface area (6).

More recently attempts have been made to use the arterial blood pressure in the standardization of hemorrhagic shock in the rat (1, 2). This paper is concerned with a method for the production of standardized hemorrhagic shock in the rat using blood pressure as the criterion. The method resembles the reservoir technique developed by Wiggers and Werle (8) for the dog, with which so much information of the cardiovascular effects of hemorrhagic shock has been obtained. The blood pressure of the rat is reduced to a desired hypotensive level and maintained at this pressure as long as desired. This allows some of the cardiovascular aspects as well as the metabolic aspects of hemorrhagic shock to be studied in the rat, an animal which can be obtained in a single strain of known history, and at a relatively constant weight. Thus variations due to heredity, environment, nutrition, etc. can be reduced.

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Contribution from the Department of Physiology, Faculty of Medicine, University of Western Ontario, London, Canada. The investigation was supported by a grant from the Defence Research Board of Canada (Grant No. 298).

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General Method

Male Sprague-Dawley rats weighing approximately 400 gm. were used. They were not deprived of food or water before anesthesia.

As shown in Fig. 2, nembutal was administered intraperitoneally (4 mgm. per 100 gm. body weight) 45 min. before the initial hemorrhage. Anesthesia usually occurred within five minutes (*A*), and then a rectal thermometer was inserted. The jugular vein and the carotid artery were cannulated and heparin was administered by injection into the carotid artery (2.5 mgm. in 0.1 ml. distilled water). A pressure system was connected to the jugular cannula which then permitted the withdrawal of blood from the vessel into a graduated reservoir from which the volume withdrawn could be recorded.

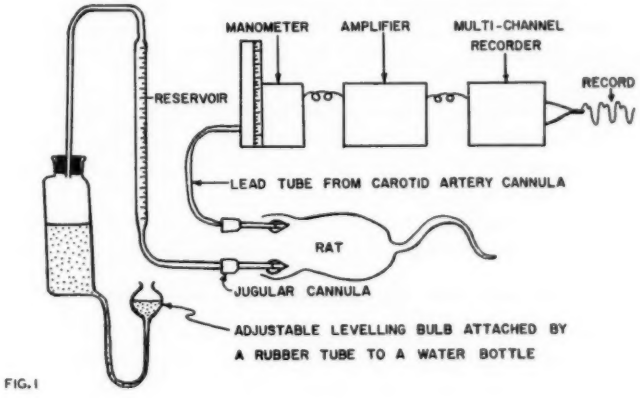

FIG. 1

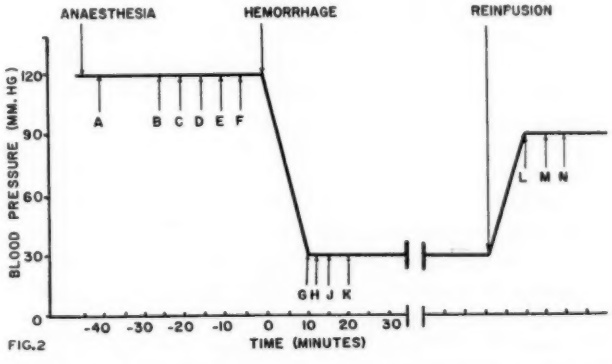

FIG. 2

FIG. 1. Diagram showing the basic instrument arrangement for the production of hemorrhagic shock in the rat.

FIG. 2. Diagram illustrating the procedure followed during the production of hemorrhagic shock in the rat.

When the levelling bulb of the pressure system was lowered, blood entered the reservoir. When the bulb was raised, reinfusion occurred. The mean arterial blood pressure was measured with a Sanborn Electromanometer and recorded with a Sanborn Multichannel recorder (Fig. 1).

The first record of blood pressure was made at *B* (Fig. 2). The respiratory rate was recorded before anesthesia, at *A*, *B*, *C*, *D*, *E*, *F*, and at the time of hemorrhage, along with the rectal temperature, blood pressure, and heart rate.

The initial hemorrhage was begun at 0 time and completed in 10 min., when the blood pressure reached 30 mm. Hg. The initial bleeding volume (IBV) was recorded at *G*. Starting at *H*, the secondary bleeding volumes (SBV) were noted every two minutes. The blood pressure was maintained at 30 mm. Hg by further withdrawals or reinfusions of blood. The rectal temperature was recorded at the end of the initial hemorrhage and five minutes later at *J*. The temperature was then recorded every 10 min. Respiratory rate, carotid artery pressure (systolic, diastolic, and a mean), and heart rate were recorded immediately after the initial bleeding and at 10-min. intervals thereafter (*K*).

At the time of reinfusion all of the indices were recorded. The blood was usually returned over a 10-min. interval, or longer if there was an indication of cardiac arrhythmia. The indices were recorded at appropriate intervals following reinfusion (*L*, *M*, *N*).

For survival studies the cannulae were removed, and the vessels were ligated. The animals which lived for 48 hr. were considered survivors.

Application of the Method and Results

In many methods for studying hemorrhagic shock a mortality rate of 80% in the "untreated" controls is considered statistically desirable to permit the clear demonstration of the efficacy of any "treatment". Previous work had shown that in the dog a blood pressure of 45 mm. Hg for 90 min. produced an irreversible type of hemorrhagic shock in 75% of the cases (7, 9). With these values as a guide, a satisfactory combination of pressure and time were sought for in the rat. Conditions used and the results obtained are shown in Table I.

A blood pressure of 45 mm. Hg maintained for 45 min. or as long as 100 min. did not produce a satisfactory mortality rate. Another approach was then attempted. Experience had shown that, in the dog, the onset of automatic reinfusion (exhaustion of the compensatory mechanisms during hemorrhage) indicated that the animal was in irreversible hemorrhagic shock. An experiment was carried out to determine this point in the rat. The blood pressure was maintained at 45 mm. Hg until one-quarter of the maximum bleeding volume had returned to the animal (automatic reinfusion). When this point was reached, the blood remaining in the reservoir was reinfused (final reinfusion). The results of experiment 2 (Table I) demonstrated that the rat could, by this criterion, withstand a hypotensive level of 45 mm. Hg blood pressure for a much longer period than the dog. It was considered that automatic reinfusion would occur sooner if the blood pressure were maintained at a lower level.

TABLE I

Expt. No.	No. of rats	Hypotensive blood pressure, mm. Hg	Hypotensive time from start of hemorrhage, min.	Survivors	Fatalities	Incomplete	% Mortality excluding incomplete group	% Mortality including incomplete group
1*a*	1	45	45	1	0	0	—	—
1*b*	1	45	75	1	0	0	—	—
1*c*	1	45	105	0	1	0	100	—
1*d*	9	45	90	7	2	0	22.2	—
1*e*	2	45	100	2	0	0	100	—
2	1	45	270	0	1	0	50	—
	1	45	252	1	0	0		
3	20	30	Until ½ MBV reinfused	2	11	7	84.6	90
4	32	30	Until time of automatic reinfusion	6	21	5	77.8	81.2

In experiment 3 (Table I) blood pressure was maintained at 30 mm. Hg until one-quarter of the maximum bleeding volume had returned to the animal. Twenty animals were used: 2 survived, 11 died, 7 were designated incomplete because the animals died before the final reinfusion was completed. The mortality of this series, excluding the incomplete group, was 85%. The mortality rate for the entire group was 90%. It appeared, then, that irreversible shock made its appearance around the time when automatic reinfusion began.

In order to reduce the number of incomplete experiments, it was decided to return all of the blood in the reservoir to the rat as soon as it could be determined that vascular failure had begun. The criteria adopted for this purpose were:

1. A reduction in the volume of blood in the reservoir at three successive two-minute intervals, while the blood pressure was maintained at 30 mm. Hg (automatic reinfusion); in some animals, however, the failure was so precipitous that other criteria were required;
2. A sudden drop in blood pressure followed by respiratory failure; or
3. The appearance of excessive excursions in the blood pressure record.

In experiment 4 (Table I) any one of these three criteria was used for terminating the period of hypotension. In this series of 32 animals, 6 survived, 21 died, and 5 were incomplete. The mortality rate, excluding the incomplete group, was 78% and for the entire group 81%. It was decided to adopt the method, level of hypotension, and criteria of its duration used in experiment 4 as a standard procedure.

A statistical analysis of the results obtained with the standard procedure (experiment 4) is shown in Table II.

Observations

The mean values of the control observations before hemorrhage on the 61 rats (408 gm. mean weight) used in experiments 1*d*, 3, and 4 were: respirations, 74 per minute; arterial blood pressure, 142 mm. Hg; and in experiment 4: heart rate, 382 beats per minute; rectal temperature 35.0° C.

Bleeding Volumes

In experiment 1*d* the initial bleeding volume (IBV) required to lower the blood pressure to 45 mm. Hg in 10 min. was 4.3 ml., and little more had to be removed to lower it to 30 mm. Hg; the average amount of blood removed in experiments 3 and 4 was 4.5 ml. Following this initial bleeding, small amounts of blood had to be removed from the animal to maintain its blood pressure at 30 or 45 mm. Hg. This blood was in a sense squeezed out of the animal by the compensatory vasoconstriction. This amount of blood was called the secondary bleeding volume (SBV). When the blood pressure was maintained at 30 mm. Hg about 0.2 ml. of blood on the average was added to the SBV every two minutes until a maximum amount of blood was removed.

TABLE II

STATISTICAL DATA, EXPERIMENT NO. 4

	Incomplete	Fatalities	Survivors
Number of rats	5	21	5
Body weight (gm.)	400 ± 16.6*	407 ± 20.9	402 ± 9.0
Initial blood pressure	143 ± 26.6	136 ± 12.3	145 ± 11.0
Final blood pressure	—	91.0 ± 22.2	106.5 ± 10.9
IBV/100 gm. body weight (cc.)	1.23 ± .19	1.19 ± .26	1.15 ± .16
SBV/100 gm. body weight (cc.)	1.02 ± .79	1.17 ± .75	1.23 ± .46
MBV/100 gm. body weight (cc.)	2.25 ± .17	2.38 ± .44	2.35 ± .40
Beginning of automatic reinfusion (min.)	54 ± 28	67 ± 25	71 ± 23
Time required for final reinfusion	—	10 ± 3.7	13 ± 3.2
Heart rate before hemorrhage	404 ± 22	364 ± 57	379 ± 24
Heart rate after hemorrhage	277 ± 41	261 ± 56	274 ± 29
Heart rate at reinfusion	298 ± 58	280 ± 55	274 ± 29
Heart rate at complete reinfusion	—	273 ± 13	315 ± 33
Respiration after anesthesia	84 ± 8.2	79 ± 7.8	77 ± 7.2
Respiration at hemorrhage	77 ± 11.7*	73 ± 11.3	72 ± 4.7
Respiration after initial hemorrhage	69 ± 14.9	72 ± 21.5	78 ± 13.0
Respiration before reinfusion	36 ± 5.1	39 ± 7.9	47 ± 6.3
Rectal temp., ° C., at hemorrhage	35.1 ± .7	35.3 ± .6	34.8 ± .4
Rectal temp., ° C., after initial hemorrhage	34.7 ± .6	34.8 ± 2.2	34.1 ± .45
Rectal temp., ° C., at final reinfusion	32.1 ± 1.3	32.6 ± 1.2	31.5 ± .9

* *Standard deviation.*

This amount, the IBV plus the SBV, was called the maximum bleeding volume (MBV). The average maximum bleeding volume for the various experiments was 9.5 ml. This maximum vasoconstriction may be maintained some minutes but soon fails, and vasodilation with spontaneous reinfusion begins, the blood pressure remaining at 30 mm. Hg (Fig. 3).

The bleeding volumes per 100 gm. of body weight for the three groups of animals in experiment 4 were remarkably similar (Fig. 4). There was a slight tendency for the initial bleeding volume to be larger in the fatalities and incomplete group than in the survivors. Observations of the individual

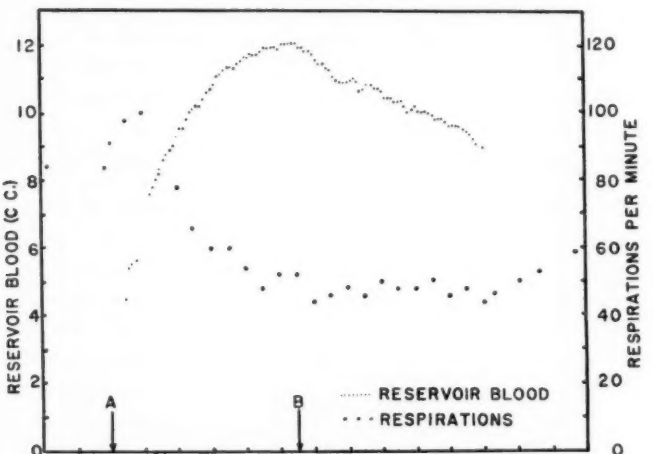

FIG.3

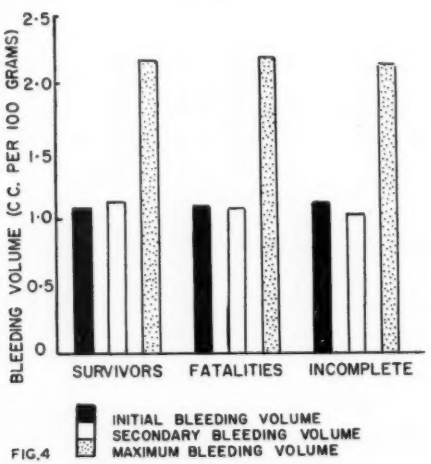

FIG.4

FIG. 3. (Experiment 3, rat No. 15.) Illustrates the changes that occur in the reservoir blood volumes and respiratory rate during hemorrhage. The hemorrhage is commenced at *A*, and automatic reinfusion begins at *B*.

FIG. 4. (Experiment 4.) Showing the variations in the bleeding volumes.

animals' reactions indicate that the initial hemorrhage may, in some way, have affected the final outcome of the procedure in each case. Although the hemorrhage was completed in 10 min., some animals refused to drop their blood pressure readily, while others showed a smooth decline throughout the period.

Respiration

The respiratory pattern of the rat in this final procedure was remarkably regular. The respiratory rate of the 400 gm. rats in the animal colony was 70 to 80 per minute. Following the production of anesthesia the respirations were about 80 per minute and remained close to this level until hemorrhage.

There was a difference in the respirations of the three groups of animals in experiment 4 during the hypotensive period. There was a tendency for the respirations of the incomplete group, i.e. those which died before reinfusion was completed, to be higher than for those of the survivors or fatalities immediately after hemorrhage. Both the fatality and incomplete groups showed a steady decline in respirations throughout the experiment, while the survivors showed an increase in the respiratory rate following the initial hemorrhage.

None of the animals showed a tendency for automatic reinfusion before the respirations had dropped to 55 per minute. The respiratory rates immediately before reinfusion were different in the three groups. The survivors did not decline as low as either the fatalities or the incomplete group; the incomplete group showed the lowest respiratory rate. The extent to which the respirations were depressed and the length of time that they remained at this low level appeared to influence the recovery of the animal following reinfusion. The lower the rate of respiration at the time of reinfusion, the less likely it was that the animal would recover.

Upon reinfusion, respiration became irregular and tended to increase; at the completion of reinfusion the rate was about 55–60 per minute. Following reinfusion the rate returned to normal or higher.

In some of the shocked animals respiratory failure occurred late in the hypotensive period. Although respiration was usually slow (45–35), automatic reinfusion had not yet occurred. If, on the development of respiratory failure, reinfusion was immediately begun, respiration returned in some of the animals, and transfusion could be completed. Those in which spontaneous respiratory action was not re-established were considered incomplete.

The respiratory rate and rectal temperature were followed in a series of animals anesthetized but upon which no surgery was performed. Most were well out of anesthesia at the end of 45 min., and all were moving spontaneously within 62 min. after the administration of the nembutal. Of these 14 animals, only 3 showed a slight decline in respiratory rate; in the other 11 respiration remained essentially unchanged.

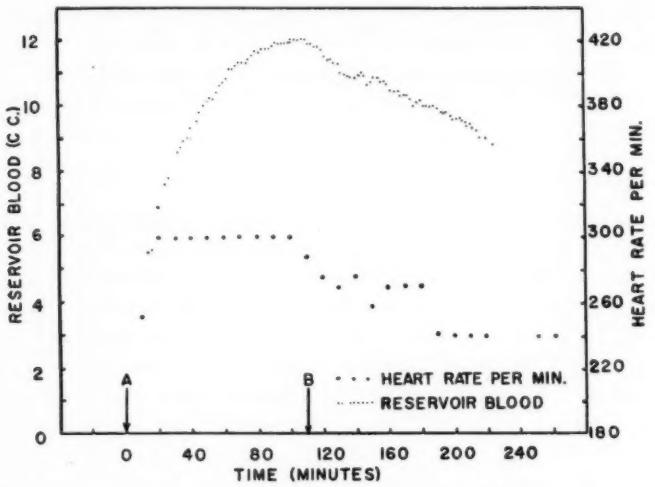

FIG. 5. (Experiment 3, rat No. 15.) Illustrates the changes that occur in heart rate during hemorrhage. *A* is the beginning of hemorrhage; *B* is the beginning of automatic reinfusion.

Heart Rate

The heart rate of these rats following anesthesia was around 382 beats per minute. Upon hemorrhage it slowed to around 260 beats per minute and then slowly increased during the early part of the hypotensive period until a plateau of around 300 beats per minute was achieved. This was maintained until automatic reinfusion commenced, at which point the heart rate again fell (Fig. 5). Following the completion of the final reinfusion the heart rate slowly returned to normal. The rate, taken from the carotid pulse, rarely showed any variation in its regularity. Occasionally, when the reinfusion was too rapid, there were indications of skipped beats.

There were variations in the heart rate of the three groups in experiment 4. The incomplete group had the fastest heart rate immediately before hemorrhage. The survivors were able to increase their heart rate towards the normal level after the completion of the final reinfusion whereas the fatalities showed a decline.

Blood Pressure

The average blood pressure of the rats in experiment 4 following anesthesia was 139 mm. Hg. During the 10-min. period of hemorrhage the blood pressure dropped slowly, and at no time were there indications of cardiac irregularity. If too much blood was withdrawn and some had to be returned to maintain the pressure at 30 mm. Hg, there was a tendency for waves of a Hering-Traube nature to appear. These disappeared when the blood pressure stabilized itself at the 30 mm. mark. In a few animals sudden fluctuations in

blood pressure occurred late in the hypotensive period; these were taken as an indication of failure to maintain a constant vasomotor tone, and reinfusion was immediately begun. In experiment 3, where the blood pressure was maintained at 30 mm. Hg until one-quarter of the maximum bleeding volume had spontaneously returned, the average blood pressure immediately after the final reinfusion was 87 mm. Hg, and in experiment 4, where one of the three criteria was used to terminate hypotension, the blood pressure returned to an average of 95 mm. Hg, but in neither experiment did the blood pressure return to normal. Usually, a considerable quantity of blood had to be given before the pressure started to rise. On some occasions after a brief increase it fell and continued to do so even though transfusion was increased in rate; such animals usually died before the transfusion could be completed.

Comparison of the individual carotid artery pressure tracings before and after the initial hemorrhage shows that the amplitude of the incisural notch had decreased considerably.

Body Temperature

The rectal temperature of the rat during the hypotensive periods tends to drop progressively until the final infusion commences, following which the temperature returns to above the prehemorrhage level (Fig. 6). The rats showed a decline in body temperature from an average of 35° C. in the prehemorrhage period to 32° C. at the end of hypotension. There was a significantly lower rectal temperature in the surviving group when compared with that of the fatalities immediately after the initial hemorrhage and immediately before reinfusion. Further observations are necessary to determine whether this difference is consistent.

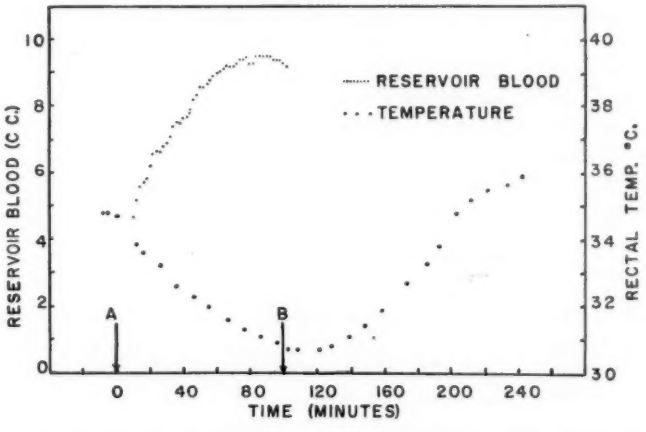

FIG. 6. (Experiment 4, rat No. 9.) Illustrates the changes that occur in rectal temperature before and during hemorrhage and after reinfusion. Hemorrhage commences at *A*, and the final reinfusion occurs at *B*.

Postinfusion Observations

The animals which revived rapidly and regained their normal posture usually survived for longer periods of time. The rats which survived the hypotensive period appeared to be divided into two main groups—those that survived for a period of time without showing any neurological symptoms, and those animals which revived, assumed normal posture and movements, and yet had a tendency to convulse. There was a tendency for the animal to roll on its side, and after recovery there appeared to be a compulsory circling movement. It was necessary to tie off the left carotid artery following reinfusion. Since the rolling and compulsory movements were towards the left, it is suspected that the occlusion of this vessel may have contributed to the convulsion. The convulsions lasted only a few seconds, and recovery occurred within two or three minutes. Within 20 min. the animal might become restless again, and on many occasions a loud noise or handling of the animal or its cage produced a second convulsion. The majority of animals showing convulsions did so at intervals for about 9 to 10 hr. (maximum 12 hr.) and died following or during one of the convulsions. Two animals convulsed at intervals and then recovered. The accumulation of a metabolic product or the production of a transient anoxia in some part of the central nervous system may be responsible for the convulsion.

Those animals which recovered quickly and were soon moving about the cage usually showed no gross lesions upon eventual post mortem. A large number of animals dying before 48 hr., especially the animals of experiment 3, showed hemorrhage into the intestine. Such animals remained prone for a long period of time. The hemorrhages occurred mainly in the lower ileum, and, if the animal lived long enough, the lesion spread upwards through the bowel and finally included the jejunum; the last one and a half inches of ileum usually showed no gross signs of hemorrhage. In a few isolated cases petechial hemorrhages were found in the stomach, especially in the lower fundic region just above the pylorus, and in the cecal wall. Intestinal hemorrhage was often copious and in some animals amounted to 5 cc. of blood, which was usually quite fluid. No hemorrhage was seen in any animal in any portion of the bowel which did not contain ingesta.

Post mortem-examination also showed congestion of the lungs and liver in approximately one-half of the fatalities.

Conclusions

1. Using a reservoir system a standardized method of hemorrhagic shock has been developed for the rat. The blood pressure is reduced to 30 mm. Hg in 10 min. and maintained at this level until there is an indication of vascular collapse, at which time reinfusion is begun.

2. Considering those animals which lived 48 hr. as survivors, it was found that in a series of 32 animals, 21 died, 6 survived, and 5 died before the completion of reinfusion. The mortality rate excluding this last group was 78%.

3. The respiratory pattern of the rats during the hypotensive period was considered to be a good indication of the probability of recovery following reinfusion. The respiratory rate declined progressively during the hypotensive period. The lower the rate of respiration at the time of reinfusion, the less likely it was that the animal would recover.

4. The heart rate declined during the initial hemorrhage and remained lower than normal throughout the hypotensive period. There were no indications of cardiac irregularities.

5. The blood pressure did not return to the original level immediately following the completion of reinfusion. In the experiment employing the standardized procedure, the average pressure following the completion of reinfusion was 95 mm. Hg as compared to a prehemorrhage level of 139 mm. Hg.

6. The average bleeding volumes per 100 gm. of body weight for the survivors, fatalities, and the incomplete group were remarkably similar. There was a tendency for the initial bleeding volume to be larger in the fatalities and the incomplete group than in the survivors.

7. The rectal temperature showed a steady decline during the hypotensive period. After the final reinfusion the temperature returned to the prehemorrhage level.

8. After reinfusion a number of animals convulsed.

9. Intestinal hemorrhage was noted in a number of animals at post mortem. The jejunum was more often involved than any other section of the bowel.

Acknowledgments

The authors are indebted to Dr. C. W. Gowdey for his helpful criticism and advice. Dr. J. W. Pearce and Dr. J. E. Merriman gave advice on special methods. Dr. T. L. Jones, principal of the Ontario Veterinary College, and Dr. H. T. Batt, head of the Department of Physiology, generously provided a leave-of-absence (H. G. D). Valuable technical assistance was given by Mr. L. Schiratti.

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DEPOLYMERIZATION OF DEXTRAN BY HYDROGEN PEROXIDE[1]

By Irving Levi and Ezra Lozinski

Abstract

It was observed that when very dilute solutions of hydrogen peroxide were employed for the purpose of destroying pyrogens in aqueous dextran solutions, a marked depolymerization of the polysaccharide molecule occurred. Further study demonstrated this effect to occur over a wide range of temperature, pressure, time, and concentration of hydrogen peroxide. It was therefore possible to use hydrogen peroxide in place of the usual hydrolytic agents, such as acids, for the depolymerization of native dextran to smaller molecular weight fragments suitable for use as a blood plasma extender.

During a study of the action of dilute solutions of hydrogen peroxide on the destruction of pyrogens (2) in 6% dextran solutions (to be used for intravenous injections), it was observed that, in addition to elimination of pyrogens, there was a marked lowering of the viscosity of the solution indicating a decrease in the size of the dextran molecule.

A more detailed study revealed that this effect was obtained over a wide range of temperature, pressure, time, and concentration of hydrogen peroxide. The small amounts of hydrogen peroxide employed had a negligible effect on the gross structure of the dextran fragments formed in this depolymerization, as indicated by optical rotation measurements and periodate oxidation. The possibility suggested itself, therefore, of utilizing hydrogen peroxide, instead of the usual hydrolytic agents such as acids, for the depolymerization of native dextrans to smaller molecular weight fragments suitable for use as a blood plasma extender.

Earlier observations have been reported on the hydrolytic and oxidative effects of hydrogen peroxide on polysaccharides other than dextran. In most of these reports the concentrations of hydrogen peroxide employed were very much greater than those used in this investigation. However, in those cases where the dilutions of hydrogen peroxide employed approached the low concentration used in this work it is significant that hydrolysis rather than oxidation was the first effect observed (6, 1).

Gatin-Gruzewska (4, 5) claimed that under the influence of weak solutions of hydrogen peroxide, at ordinary temperatures, polysaccharides such as starch and glycogen undergo both hydrolysis and oxidation. Gerber (6, 7) has similarly stated that very dilute solutions of hydrogen peroxide act as powerful hydrolyzing agents for starch, producing maltose and dextrins. In general, the time – temperature – hydrogen peroxide concentration relationship found by Gerber for starch is the same as that found for dextran. This hydrolysis of starch, in which hydrogen peroxide is assigned the role of a catalyst, is followed at higher hydrogen peroxide concentrations, by an

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Contribution from the Research Laboratories, Charles E. Frosst & Co., Montreal, Quebec, Canada. This work was reported at the Eighteenth Annual Meeting of the Canadian Physiological Society, Toronto, October, 1954.

oxidation of the maltose previously formed, accompanied by decomposition of the hydrogen peroxide. Both Gerber and Gatin-Gruzewska claim that the hydrolysis obtained from the action of hydrogen peroxide on starch solutions more nearly resembles diastatic saccharification than acid hydrolysis. Durieux (3) using hydrogen peroxide and ferric chloride on starch similarly concluded that the action was analogous to that produced by diastase.

Neuberg and Miura (10) tested hydrogen peroxide, in relatively strong concentrations, for its hydrolytic properties toward large molecular compounds such as proteins, starch, inulin, and yeast nucleic acid and concluded that in all cases hydrogen peroxide manifested, in addition to its oxidative action, distinct hydrolytic properties. These authors postulated that since hydrogen peroxide very probably appears as an intermediate in plant and animal substances and is able at ordinary temperatures to effect profound hydrolytic splitting, hydrogen peroxide may exert an influence in the organism in connection with catalase. In this connection it is interesting to note that Sieber (13) also considered dilute solutions of hydrogen peroxide as an admirable hydrolyzing agent for proteins such as casein, hemoglobin, keratin (human hair), hemin, and tubercle bacillus.

Brown (1) concluded that the action of hydrogen peroxide and ferrous sulphate (Fenton's reagent) upon starch is a hydrolysis, producing, in the course of the reaction, dextrins, sugars of high molecular weight, and simple sugars. He claims that the reaction is analogous to that produced by amylase, differing only in the fact that the simple sugars produced are further hydrolyzed and oxidized to acids and aldehydes.

Various other workers have reported the hydrolysis of carbohydrates by hydrogen peroxide. Schonebaum (12) showed that heating neutral solutions of sucrose in the presence of even less than 0.15% hydrogen peroxide gave considerable inversion. Starch solutions have been hydrolyzed to water soluble dextrins by the action of dilute hydrogen peroxide solutions (11, 15, 16). Hydrogen peroxide even in concentrations as low as 0.0002 *M* decreased the viscosity of solutions of hyaluronic and chondroitin-sulphuric acids (14). Kertesz (9) observed that dilute solutions of hydrogen peroxide rapidly degraded dissolved pectin whereby the pectin lost its typical colloidal properties. He suggested that some of the changes that take place in the polysaccharides and pectic constituents of plants *in situ* may be the result of the action of peroxides on these substances.

In the present paper various examples are given of the depolymerization of native dextran with dilute solutions of hydrogen peroxide, and the nature of the resulting products described.

Experimental

The dextran used in this work was produced from sucrose by the action of *Leuconostoc mesenteroides* NRRL-B-512[2] following the procedure given by Jeanes, Wilham, and Miers (8).

[2] *Kindly supplied by Dr. Allene Jeanes of the Northern Regional Research Laboratories, Peoria, Illinois.*

All relative viscosity measurements reported were determined at 25° C. using an Ostwald viscometer having a water flow time of 80–100 sec.

Example 1.—A series of native dextran solutions were prepared which contained either 1%, 6%, or 12% dextran (w/v) and 0.10, 0.02, 0.01, or 0.001 *M* hydrogen peroxide. All of the solutions were very viscous, and opalescent. The depolymerizations were carried out under varying conditions of temperature, pressure, and time. During the depolymerization all the solutions became very fluid and nearly water-clear in color. The relative viscosity of each solution was determined. Table I summarizes the experiments.

TABLE I

EFFECTS OF HYDROGEN PEROXIDE CONCENTRATION, PRESSURE, TEMPERATURE, AND TIME ON THE RELATIVE VISCOSITY OF DEXTRAN SOLUTIONS

% Dextran	Molar H_2O_2	Depolymerization conditions			Rel. viscosity of depolymerized soln.
		Pressure	Temp. ° C.	Time	
1	0.01	5 p.s.i.	109	5 Min.	4.80
12	0.10	5 p.s.i.	109	5 "	18.36
1	0.001	Atmospheric	100	30 "	1.96
6	0.01	"	100	30 "	3.83
12	0.01	"	100	30 "	21.5
12	0.02	"	100	30 "	8.68
6	0.01	"	25	119 Hr.	36.4
6	0.01	"	25	146 "	14.7
6	0.01	"	25	172 "	9.2
6	0.10	"	25	119 "	2.04
1	Nil	—	—	—	6.35
6	"	—	—	—	760
12	"	—	—	—	Too viscous for Ostwald viscometer

Example 2.—A series of dextran solutions were prepared by dissolving 1.2 gm. of native dextran in distilled water and adding sufficient amounts of 0.1 *M* hydrogen peroxide to give final solutions containing 6% (w/v) dextran and molarities of hydrogen peroxide as shown in Table II. All of the solutions were heated in an autoclave at 15 p.s.i. (121° C.) for 20 min. The relative viscosities of the solutions were then determined and are listed in Table II.

Example 3.—A 6% (w/v) solution of dextran was prepared by dissolving 190 gm. of dextran in 3166 ml. of distilled water. This very viscous, opalescent solution had $[\alpha]_D^{25}$ $+181.1 \pm 2.3$ ($c = 1\%$, water) and a relative viscosity of approximately 760. Sufficient 30% hydrogen peroxide was added to give a final concentration of 0.01 *M* H_2O_2. The solution was heated in an autoclave at 15 p.s.i. (121° C.) for 20 min. whereby the solution became very fluid, was nearly water-clear, and had a relative viscosity of 2.80. The residual hydrogen peroxide was destroyed by the addition of a small amount

TABLE II

EFFECT OF HYDROGEN PEROXIDE CONCENTRATION ON RELATIVE VISCOSITY OF DEXTRAN SOLUTIONS

Conditions: Dextran 6% (w/v) autoclaved 15 p.s.i., temperature 121° C., for 20 min.

Solution No.	Molarity of H_2O_2	Relative viscosity of depolymerized solution
1	0.00	Approx. 760
2	0.001	82.0
3	0.002	26.2
4	0.004	8.82
5	0.006	5.94
6	0.008	5.05
7	0.01	3.38
8	0.02	2.22
9	0.03	1.85
10	0.04	1.61
11	0.05	1.57

of charcoal and warming. The solution was filtered to give a clear filtrate. The depolymerized dextran from 3000 ml. of this filtrate was fractionally precipitated by successive additions of acetone. Each fraction of dextran was dried and redissolved in 0.85% saline to give a 6% solution. The viscosity and $[\alpha]_D^{25}$ of each solution were determined. The results are shown in Table III.

TABLE III

PROPERTIES OF HYDROGEN PEROXIDE DEPOLYMERIZED ACETONE PRECIPITATED DEXTRAN FRACTIONS

Fraction No.	Acetone added, ml.	Wt. of fraction, gm.	Relative viscosity of 6% soln.	$[\alpha]_D^{25}$ (c = 1%, H_2O)
1	1375	13.0	9.48	+180.16
2	+ 150	47.7	4.55	+180.93
3	+ 100	20.4	3.53	+180.16
4	+ 200	19.7	2.84	+179.88
5	+ 300	23.2	2.44	+183.21
6	+ 500	11.6	2.00	+182.55
7	+1800	19.1	1.81	+106.15

3166 ml. of a 6% dextran and 0.01 M H_2O_2 solution autoclaved at 15 lb. for 20 min. Final relative viscosity 2.80. Acetone added as shown in table.

Discussion of Results

It will be seen from the above examples that by subjecting aqueous solutions of dextran to the action of hydrogen peroxide in such concentration as to give a final concentration equivalent to between 0.001 and 0.10 *M* hydrogen peroxide at temperatures ranging from 25° C. to 121° C., and pressures up to

15 p.s.i. for a period of time which in relation to the preceding variants may range from several minutes to several days, it is possible to depolymerize the dextran molecule.

Table I illustrates the effect on the relative viscosity of the product, and thus the extent of depolymerization, of varying concentrations of hydrogen peroxide on native dextran solutions of 1%, 6%, and 12% under varying conditions of time, temperature, and pressure. It will be seen that a low concentration of depolymerizing agent requires for the same percentage solution of dextran a comparatively long depolymerization time and/or an elevated temperature. Conversely, with a higher concentration of hydrogen peroxide the depolymerization is effected in a comparatively short time at the same temperature and/or pressure.

Table II shows that the degree of depolymerization obtained under constant conditions of temperature, pressure, time, and concentration of dextran solution is dependent on the molarity of the hydrogen peroxide employed.

It will be seen from Table III that the optical rotations of fractions 1–6 isolated from a hydrogen-peroxide-depolymerized solution of dextran are within the limits of that exhibited by the starting material. Therefore, although the dextrans in these fractions represent varying degrees of depolymerization, they still exhibit the gross structure of the starting material, and are depolymerized homologues of the original dextran. Fraction 7, which has a much lower $[\alpha]_D$, represents the highest depolymerized, and therefore lowest molecular weight material isolated, and is beginning to lose the physical properties of native dextran.

Summary

Dilute solutions of hydrogen peroxide effect a marked lowering of the viscosity of dextran solutions, indicating a decrease in the size of the dextran molecule due to a depolymerization.

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Symposium on Electrolytes

THE SIGNIFICANCE AND REGULATION OF ION DISTRIBUTION IN ANIMAL TISSUES AND FLUIDS

This symposium was held on the afternoon of October 23rd as part of the program of the 18th annual meeting of the Canadian Physiological Society, in Toronto, October 22 and 23, 1954. Dr. R. A. Cleghorn of McGill University, Montreal, acted as Chairman of the Symposium, which was organized by Dr. J. F. Manery of the University of Toronto.

GENERAL PRINCIPLES AND PROBLEMS OF ELECTROLYTE RESEARCH[1]

By J. F. Manery

The term "electrolytes" was applied by Faraday to the substances which were lyzed or loosened by the passage of an electric current. The process was electrolysis and the bodies which passed to the anode he called anions, and those to the cathode, cations. Together they were referred to as "ions", the Greek for wanderer. To Arrhenius we are indebted for broadening these ideas and for the current view that ions exist in electrolyte solutions and move when an electric current is applied. This Electrolyte Symposium will deal with certain ions of importance in biology—chloride, bicarbonate, phosphate, calcium, magnesium, potassium, and sodium. It must be borne in mind that, when reference is made to sodium chloride, sodium bicarbonate, or potassium phosphate, a terminology of ancient origin is employed which is meant only to imply that some sodium might be matched by chloride or bicarbonate and is not meant, in any sense, to suggest that the salt sodium chloride exists in the solution.

It is unfortunate that the limitations of time do not permit a brief sketch of the historical development of electrolyte research (see (19)), because there is no better way of illustrating to you the increasing importance which the inorganic ions are now assuming in many biological problems. Since the early eighteen hundreds when meat was analyzed by chemists for its electrolytes because it was an important food, research in this field progressed rather slowly until the last decade. Then, as in every other field of biochemistry, the use of isotopes revolutionized the experimental approach to electrolyte research. The study of the metabolic roles of enzymes and of phosphorus compounds advanced rapidly and chapters on intermediary metabolism had to be entirely rewritten. And so the concept of the static function of the inorganic ions of maintaining osmotic pressure and electroneutrality and of providing a suitable medium for proteins, along with the idea of the relatively impermeable membrane has given way to a much more

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Contribution from the Department of Biochemistry, University of Toronto, Toronto, Canada. This paper was presented at the Symposium on Electrolytes held as part of the Annual Meeting of the Canadian Physiological Society, Toronto, Ontario, October 22 and 23, 1954.

dynamic picture. Although all cells maintain a characteristic concentration of inorganic ions, these ions traverse membranes continuously at a great variety of rates; they accelerate and inhibit enzyme-controlled reactions; indeed, the very movements of the inorganic ions themselves seem now to be closely linked to metabolic processes. Instead of picturing biochemical processes as taking place in a continuous aqueous medium of physiological salt solution, it has been suggested that some of the inorganic ions too, like the enzymes, are strategically located at specific sites throughout an organized cell and so the research is progressing to a study of electrolytes in cell inclusions such as nuclei, mitochondria, microsomes, etc. (1).

In the remarks to follow I hope to describe what seems to me to be the fundamental principles of electrolyte research and, in discussing the attempts to establish them, some of the problems encountered will be evident. Wherever possible I shall use muscle as my example firstly, because an organized tissue presents problems which cannot be solved using yeast cells, algae, bacteria, or red cells, and secondly, because other cell types will be considered elsewhere in this program.

Principles

1. Electroneutrality

According to the law of electroneutrality the sum of the concentrations of all cations must equal the sum of the concentrations of all anions, concentrations being expressed in equivalents or milliequivalents (not grams or milligrams) per liter or kilogram of water. The determination of the total cation content, although tedious and difficult, is an important measurement to make, because it tells you at once the *anionic* concentration and the total concentration of ion equivalents. The term "total base" has long been applied to this measurement and, although incorrect in the new sense that a base is a hydrogen acceptor, the term is in common use throughout biochemical, physiological, and clinical literature.

In order to demonstrate the law of electroneutrality analyses of the substances contributing anions must be made, and the number of anion equivalents which they provide per mole (for matching the cations) must be calculated. Correct estimation of the number of anions provided per mole by proteins and by bicarbonate and phosphate esters depends on the solution of two problems, (*a*) finding the pH of the medium and (*b*) determining the dissociation constants of the weak acids. The fluids of the body present no difficulty because the anions are mostly chloride and bicarbonate and the pH is easily determined. Since there is no entirely satisfactory direct method of determining intracellular pH and since the dissociation constants of many of the organic compounds inside cells are quite unknown, the two problems mentioned above are of major significance in electrolyte research.

That some advance toward the solution of these two problems has been made is attested by the equality of the anion equivalents and cation equivalents in red cells. The bulk of the anions are supplied by chloride and

bicarbonate but in dog red cells about 40 meq. per liter of water were calculated to be provided by hemoglobin and 30 by diphosphoglycerate (total cation concentration, about 160 meq. per liter of water). These values were based on recent estimations of the anionic equivalency of hemoglobin and of diphosphoglyceric acid, and on an internal pH of 7.17, calculated from the Henderson–Hasselbach equation for carbonic acid using the estimations, most recent at that time, of carbamino compounds and of the pK_1 value for this equation (24, 25).

Brain differs from red cells in having such a high concentration of inorganic cations that there is a large deficit of known anions, i.e. those provided by chloride, bicarbonate, and phosphate esters (18). In invertebrate nerve this deficit appears to be satisfied by amino acids (30) but Folch-Pi suggested (10) that in the brain the deficit could easily be provided by acidic phosphatides and sulphatides.

Dubuisson (8) has made what appears to be a successful attempt at showing equality of anion and cation concentrations in muscle. By accepting an intracellular pH value of 7.20 (obtained by microinjection of indicators) and using the published pK values or titration curves of creatine, carnosine, orthophosphates, phosphagen, adenosinetriphosphate, myosin, and myogen, he calculated the total anion content to be 210 meq. per kgm. of muscle. To the sum of the inorganic cations of 173 meq. per kgm. of muscle he added the cations contributed by creatine (7 meq.), by carnosine (14 meq.), and by acid amides (9 meq.) making a total of 203 which is remarkably close to the total anion value. Others have made similar attempts ignoring the organic bases because so little is known about them. The indicator method of determining intracellular pH has been criticized because of the acidity produced by the injury. A value of 6.9 has been calculated from the Henderson–Hasselbach equation for the bicarbonate system, but the calculated pH will be lower if any appreciable amount of carbamino carbon dioxide exists in the muscle fiber (see (19) for references).

2. Osmotic Equality

In the animal organism where water is known to pass easily into and out of cells and from one fluid to another the view has long been held that the same osmotic pressure exists throughout. The freezing point depressions of many, though not all, body fluids have been shown to be almost identical with that of plasma. When you express the total concentrations of the components in moles or millimoles per liter of water of these body fluids you get the same value as that in serum.

Before osmotic equality can be shown between cells and their environment in an organized tissue like muscle or liver, the concentrations inside the cells must be known. Up to the present time the cells of tissues have never been separated for analysis from the extracellular fluid and solid structures in the extracellular phase. Only an entire block of tissue, including all of its components, has been analyzed. Many investigators have accepted the view

that muscle fibers are free of chloride and that in a resting muscle all of the chloride is confined to the extracellular water. If its concentration there is known, then the volume of extracellular water may be calculated as follows[2]:

$$(H_2O)_E^{Cl} = \frac{(Cl)_T}{(Cl)_E} \times 1000.$$

Others (3) assumed that the chloride ions were distributed between the intra- and extra-cellular phases according to a Gibbs–Donnan equilibrium, and preferred to use inulin to measure the volume of extracellular water. Knowing this volume and considering the ion concentration there to be the same as in plasma, the amount of ions in the extracellular phase can be calculated. If this amount is now subtracted from that found by analysis in a block of tissue then the remainder represents the quantity of ions existing inside the cells (19).

Before this principle of osmotic equality can be demonstrated the problems to be solved are clear: (*a*) how to accurately estimate the concentrations both inside and outside of cells, and (*b*) how to obtain effective molecular weights of many organic compounds, and (*c*) how to estimate ionic activities of inorganic ions so that these values rather than concentrations can be used to calculate osmolar concentrations.

Applying the best information available at the time, the concentrations of osmotically active entities inside and outside of muscle cells have been calculated for frog and cat and a good approximation of osmotic equality was reached. This and further evidence of osmotic equality across cell walls has been summarized elsewhere (19). Also the more recent suggestion was reviewed that tissue cells are not isosmotic with their environments but are hypertonic (27) and that energy is required to continuously pump the water out, which enters in response to this hypertonicity. The reasons for this suggestion were (*a*) that tissue slices swell when placed in supposedly isotonic and even in hypertonic media, (*b*) that chilling and cyanide which interfere with metabolic processes cause swelling, and (*c*) that freezing point and melting point depressions of ground frozen tissues are less than those of plasma (5, 23). The matter is still unsettled and will probably remain so until detailed analyses prove that in soaked tissue slices, in those chilled or exposed to cyanide or in a thawing, ground tissue mass, the cell permeability of the intact tissues has not been changed or the number of osmotically active constituents in all the preparations artificially increased.

3. Electrolyte Inequality

Many problems in electrolyte research arise because of the fact that both the kind and concentration of ions inside cells differ from those in the external medium. In general, potassium is the predominating intracellular cation while sodium abounds in the extracellular medium. This description applies

[2] $(H_2O)_E^{Cl}$ = *volume of extracellular water in a kilogram of tissue.*
$(Cl)_T$ = *meq. of chloride per kgm. of tissue.*
$(Cl)_E$ = *meq. of chloride per 1000 ml. of extracellular water. This value is approximately equal to the concentration of chloride in the plasma.*

to muscle fibers, to most red cells, to liver, kidney, and brain cells and their respective environments. The ions which match the sodium outside are mostly chloride and bicarbonate, and to a lesser extent, protein, phosphates, and organic acids. Those which balance the potassium inside are made up, in muscle, largely of phosphate esters, ATP, diphosphoglycerate, hexose-phosphates, creatine phosphate, myogen, and myosin, bicarbonate, and perhaps chloride under certain conditions (20, Fig. 2). For all tissues the problem remains of determining accurately intracellular concentrations. The figure just referred to brings another unsolved problem to our attention. There is no direct method of partitioning the solids (found by analysis of whole tissue) between the extracellular and intracellular phases. This figure illustrates an attempt to use the concentration of tendon-like proteins to estimate the solids in the extracellular phase. The remaining solids were assumed to be intracellular. Not until some such procedure is used can we estimate the concentration of cell water per kilogram of cells.

It is abundantly clear that one of the most characteristic activities of living cells is this ability to maintain within cell boundaries a chemical composition differing from that of the external medium. Particularly important is the ability to concentrate potassium and extrude sodium against high concentration gradients. Potassium is 35 times as abundant inside the muscle cell as out, and sodium is about 20 times as abundant outside as in (19). The factors examined which might influence this separation of ions are four in number: (*a*) the cell membrane, (*b*) the physico–chemical properties of the ions, (*c*) the adsorption of ions onto fixed charges in the interior of the cell, (*d*) the metabolic processes which provide energy for active transport.

For over 50 years the view has prevailed that the selective accumulation of ions in muscle was a function of the *membrane*, and that the resting potential was a form of diffusion potential described by the "Ostwald-Bernstein" equation[3]. According to this theory the membrane is semipermeable, i.e. impermeable to the hydrated sodium ion and the large intracellular anions, the latter holding inside the cell the hydrated potassium ion to which the membrane is permeable. Radioactive isotopes have been used to show that both sodium and potassium can enter nearly all cells more or less readily and the former permeability constant of diffusion[4] has, where appropriate, given way to Ussing's (33) newer terms "inward flux", "outward flux", and "net flux" which do not identify in any way the process concerned. But reports are now published in which sodium and some of the so-called "impermeant" anions (proteins, carnosine, hexose phosphate esters, and amino acids) have been interpreted to enter cells (muscle, bacteria, and brain) (15) and if so the limiting membrane as previously conceived cannot possibly provide an adequate separating mechanism.

[3] *$E=(RT/F)\ln(C_1/C_2)$, where E is the diffusion potential, R, T, and F represent the gas constant, absolute temperature, and Faraday's constant, and C_1 and C_2 are the concentrations of potassium inside and outside the cell, respectively.*

[4] *The permeability constant is the amount of a substance which crosses a unit cross-sectional area in a unit time owing to a unit difference in concentration.*

According to Spiegelman and Reiner (31) none of the *physico–chemical* differences between sodium and potassium ions (polarizability, molecular weight, and ionic mobility) are sufficient to account for their separation in biological systems.

In Ling's recently proposed theory (15) of selective ion accumulation consideration has been given to the physico–chemical constants of the cations and to the character of the large intracellular anions. Ling's paper should be consulted for the details and for evidence of this theory but, in brief, he suggested that proteins might provide fixed anionic charges which would select potassium ions rather than sodium ions because of the lower dielectric constant of water between such anions and the smaller cations. Proteins such as myosin adsorb ATP, ADP, and AMP and new fixed negative charges result. Ling showed that the potential of muscle[3], which is intimately associated with the inorganic ion concentrations, was also related to the concentration of creatine phosphate, ATP, and hexosemonophosphate. Hence, as this theory is evolved, another process implicating phosphorus compounds appears, namely that of ion accumulation. Some of the synthetic ion-exchange resins show greater adsorption affinity for potassium than for sodium (2) but none of the cellular proteins have thus far been prepared in such a way that ion selectivity can be demonstrated satisfactorily. Thus, much work remains to be done before this idea has been completely explored.

The possibility too has not yet been investigated that sodium may occupy entirely different anionic sites from those occupied by potassium. I have long suspected that, although potassium lost from cells under a variety of conditions equalled the sodium gained (19, p. 381), this equality in equivalents was only fortuitous and that the ions might occupy different locations inside the cell and fulfill different functions. Early ideas worthy of re-examination are those of Mond and Netter (21) who associated the sodium of muscle fibers with the sarcolemma, of MacCallum (17) who observed potassium in the anisotropic band and of Dubuisson (8) who suggested a partitioning of the various ions in muscle between the anisotropic band of the myofibrils and the interfibrillar spaces (see also Steinbach (32)).

Whether or not the separating mechanism involves phosphorus esters as Ling suggests, there is no doubt about the fact that *metabolic processes* support ion accumulation in cells or ion movement into and out of cells. That "active transport"[5] requires energy yielding processes may almost be taken as a fourth principle in electrolyte research.

Active transport has been investigated in a great variety of cells throughout the plant and animal kingdom. In general the relation of the rate of oxygen consumption to the quantity of ions accumulated is studied and the

[5] *Active transport applies to cases where transfer takes place from a lower to a higher electrochemical potential. Not only must the ionic concentrations be known but also the electrical potential difference across the membrane (33). "Passive" transport of an ion occurs (a) if in the steady state it is distributed according to the Donnan equilibrium and (b) if the ratio between outflux and influx equals the ratio between the electrochemical activities of the ion in the intracellular and extracellular phases respectively (34).*

influence of inhibitors on these processes is observed, i.e. the inhibitors which are known to affect glycolytic reactions, oxidative and phosphorylative processes. Frequently cells or tissue slices are allowed to lose their intracellular potassium by chilling or soaking, and the factors are then studied which accelerate or inhibit its re-entrance. Attention is drawn to the fact that the loss of potassium is passive as is the coincident gain of sodium usually found in these circumstances. During recovery, however, both the potassium uptake and the sodium extrusion provide examples of active transport. If any general statement can be made it is that in yeast, bacteria, red cells, muscle, and liver, potassium uptake is probably related to glycolytic processes; in brain and kidney it may depend on oxidative processes. The distinction is not clear-cut nor is the character of the process causing the extrusion of sodium.

Two of the best examples of the relation of ion movement to cell metabolism have been demonstrated in plant cells. Carrot slices will transport potassium chloride against a high concentration gradient (16, 26). At the same time increased oxygen consumption occurred such that for every 4 gm. moles of anion transported, 1 gm. mol. of oxygen was consumed. The suggestion was made that the cytochrome system which acted as an electron carrier in one direction across the cell could act as an anion carrier in the other direction. This idea has been applied to the production of hydrochloric acid by the gastric mucosa where a high oxygen consumption accompanies active secretion (6).

Scott and Hayward using the marine algae, *Ulva lactua*, have recently provided (28, 29) a very beautiful example of the relation of ion transport to metabolism. These marine cells accumulate potassium and extrude sodium against high concentration gradients. Monoiodoacetic acid, known to inhibit phosphoglyceraldehyde dehydrogenase and thus prevent the formation of 3-phosphoglyceric acid, causes a marked loss of potassium and gain of sodium in the dark but has no effect in the light although it appears to penetrate the cells in the light (28). Since 3-phosphoglyceric acid is believed to be the first stable product formed in the photosynthetic reduction of carbon dioxide, the data suggested that the normal potassium accumulation of the cell and perhaps sodium extrusion depended in some way upon the presence in the cell of 3-phosphoglyceric acid.

In a subsequent paper Scott and Hayward (29) summarized and extended their evidence that the potassium and sodium movements in and out of *Ulva* cells were supported by metabolic processes which were to a large degree independent. For example, arsenate, believed to permit circumvention of the metabolic step inhibited by monoiodoacetic acid, protected the algae cells against the potassium loss caused by iodoacetic acid in the dark, but allowed the usual sodium uptake to occur. Moreover, the kinetics of potassium loss were distinctly different from those of sodium gain in the presence of 4,6-dinitro-*o*-cresol, an agent assumed to "uncouple" oxidative and phosphorylative processes.

Allusion has already been made to other instances where potassium accumulation is divorced from sodium extrusion. If these ions do not exchange we must look to the nature of the accompanying anion since the principle of electroneutrality must be obeyed. Carrier systems and surface enzymes have been postulated and another problem in electrolyte research is formulated.

Recognized Biochemical Actions of Electrolytes

Although the use of a salt solution for the immersion of living cells or tissues is as much of a commonplace as the use of water as a solvent, we have only the most general idea of the function of these salts either outside or inside cells. Acquiring this knowledge is the ultimate goal to which all investigators in this field strive. A survey of the following recognized actions of electrolytes demonstrates clearly that their functions are important and widespread:

1. They maintain electroneutrality and osmotic equality.
2. They influence the solubility of colloids (cell cytoplasm and membrane).
3. They influence excitability and transmission of impulses in nerve and muscle.
4. They supply physiological buffer systems.
5. They influence metabolic processes in cells, e.g. glycogen formation (12), fatty acid metabolism (11), and oxygen consumption (7, 9).
6. They influence enzymic reactions.

None of these listed actions requires elucidation with the exception perhaps of numbers 5 and 6 (recently reviewed by Lardy (14)). The evidence is impressive that all phosphorylations involving the adenylic system require inorganic cations; those involving ATP universally require magnesium ions. Calcium ions may sometimes be substituted but often, too, they inhibit. On the other hand, myosin ATP-ase is inhibited by magnesium but requires calcium. Fructokinase and phosphopyruvate transphosphorylase require potassium in addition to magnesium. Magnesium has long been considered to be a cofactor for oxidative phosphorylations and potassium too may be in some way related to this process. In general, low concentrations of the divalent ions are effective while rather high concentrations of monovalent ions are required. Sodium ions are frequently ineffective or inhibitory while potassium ions stimulate. Potassium, like magnesium (4, 13), has been shown to increase enzymatic synthesis of peptide bonds (glutathione (13)). Finally the metabolism of certain fatty acids by liver and kidney slices has been greatly increased by replacing sodium in the medium with potassium (11).

There is little to be gained by listing further the isolated enzyme systems or various metabolic processes influenced by inorganic ions. Nor is it profitable to discuss the theories, such as the formation of metalloenzymes or metallosubstrates, which have been postulated to explain this influence (22). We have been aware for some time of the relation of potassium to carbohydrate metabolism in the intact organism, in yeast, blood cells, bacteria, muscle, and brain. Too little attention has been paid in the past to the behavior of

sodium in many of the experiments performed. A study of the effect of these ions on metabolic steps related to fatty acid metabolism and protein synthesis is just beginning.

It has been the aim of this communication to present some sort of general picture of the present status of electrolyte research, as well as to emphasize a few of the simple principles which must not be ignored. Only a few of the many available references have been included, with the idea that each one listed will lead into the pertinent literature. For this reason reviews have been favored. Clearly we are entering a new era in this field of endeavor and, if we dared hope for as rapid a development as that which has recently taken place in phosphorus research, interesting days lie ahead. The solution of the problems of potassium alone may add immeasurably to our understanding of the working of a cell, when one considers the universally high concentration of the potassium inside cells, its importance in so many cell functions and now its intimate association with intracellular metabolic processes.

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DISCUSSION: ORVILLE F. DENSTEDT[1]

In the time allotted I shall be able to discuss only two or three of the fundamental points brought out in Dr. Manery's comprehensive review. Perhaps the most significant of these is the new concept of the metabolic control of the movement of ions between the tissue cells and the extracellular fluid.

Dr. Manery has pointed out that the high concentration gradient of potassium ions between the tissue cells and the surrounding fluid, and the correspondingly steep gradient of sodium in the reverse sense, are maintained by a continuous expenditure of energy by the cells. The formerly held notion that the gradients exist by virtue of impermeability of the cell mmebranes to these ions is no longer tenable. Studies with radioactive isotopes of sodium and potassium have shown that the cell membranes are freely permeable to these ions. In normal health potassium is confined largely within the cells, and sodium, largely in the extracellular fluid. It is now believed that the passage of the ions across the membrane in the living cell against the high ionic gradients is effected by a transport mechanism which operates under control of the cell's metabolism. Whether sodium only is actively transported while potassium may move by passive diffusion as a consequence of the movement of sodium has not been settled. Some authorities believe that an active transport is required in both cases. In the case of the red blood corpuscles, the energy used in driving these mechanisms is derived from glycolysis. Nothing is known about the manner in which the transport processes are coupled with the energy-producing system of the cells.

For purpose of illustration it is convenient to refer to the mammalian erythrocyte. Although this cell is, in many respects, not typical of somatic cells, it is similar in that it exists in a dynamic state and does work requiring a constant expenditure of energy. Being more amenable than other cells to quantitative study, more is known about its osmotic behavior under various conditions.

The concentrations of sodium and potassium in the erythrocytes of various species differ widely, while the concentrations in the blood plasma and extracellular fluid are remarkably uniform. Examples of the distribution of these ions are given in Table I.

TABLE I

DISTRIBUTION (MEQ./LITER) OF SODIUM AND POTASSIUM IN THE BLOOD

		Erythrocytes	Plasma	Ratio
Human	K^+	100	4.5	22 : 1
	Na^+	11	140	1 : 13
Ox	K^+	20	4	5 : 1
	Na^+	80	140	4 : 7
Dog, cat	K^+	5–10	4	2 : 1
	Na^+	100	140	5 : 7

In the case of muscle cells the distribution of potassium and sodium between the cells and extracellular fluid is indicated by the following ratios: K/Na for intracellular fluid 35/1; K/Na for extracellular fluid 1/20.

Most conditions that tend to impair the metabolic activity of the cells will cause a loss of potassium and an almost equivalent uptake of sodium. Among the conditions which may decrease or impair the glycolytic capacity of the red blood cell are the following:

(1) Lowering of the temperature, as in the storage of blood at 5° C. in the blood bank.
(2) Lowering of the pH of the external medium, as in the preservation of cells in the ACD (citric acid – sodium citrate – dextrose) medium.
(3) Depletion of glucose, as may occur when blood is kept in citrate medium without added glucose.
(4) Retardation of glycolysis by added enzyme poisons, such as oxalate, iodoacetate, or sodium fluoride.
(5) Gradual and spontaneous failure of certain enzymes of the glycolytic system, as occurs despite the presence of an ample supply of glucose on prolonged storage of red cells.

At 37° C., potassium escapes from the human red cell at the rate of about 1.5 meq./liter of cells/hr. Concurrently it is regained from the plasma at the same rate against the high concentration gradient, thus maintaining a constant concentration within the cell. The concentration gradients and equilibrium both of potassium and sodium are maintained by the energy derived from the glycolysis of glucose. The normal residual glucose content of the blood is sufficient to maintain glycolysis *in vitro* for about 10 hr. at 37° C.

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When the red blood cells are stored in the cold (e.g. at 5° C.) the permeability of the cell membrane to all substances is decreased and the rate of efflux of potassium ions is diminished to about 0.15 meq./liter of cells/hr. At the same time the metabolic (glycolytic) activity of the cells is reduced to between 1/15 and 1/25 of the activity at 37° C., with the consequence that the capacity of the cells to regain potassium and exclude sodium is greatly decreased. Potassium, therefore, is lost from the cell more rapidly than it is regained, and sodium is gained more rapidly than it can be expelled, the molar exchange of the two ions being almost equal.

The following table indicates the approximate times when the potassium of the erythrocytes in blood specimens preserved in various media at 5° C. reaches half and full equilibrium, respectively, with that of the surrounding medium (plasma). The table indicates also the approximate times when ATP (adenosinetriphosphate), the main energy store of the cells, becomes depleted during storage.

TABLE II

ESCAPE OF POTASSIUM FROM THE RED CELLS AND DEPLETION OF ATP DURING STORAGE OF BLOOD AT 5° C.

'Preservative' medium	Half equilibrium (20 meq./l.) (days)	Full equilibrium (40 meq./l.) (days)	Depletion of ATP (days)
Citrate	5	20	10–15
Citrate–glucose	12	40	25–30
ACD	23	60	45–50

It is noteworthy that the progressive failure of the cells to maintain the cationic gradient is fairly closely correlated with the failure to regenerate ATP. The concentrations of sodium and potassium reach equilibrium between the intra- and extra-cellular fluid shortly after ATP has been depleted and the capacity of the cells to maintain an ionic gradient is lost.

Information is incomplete concerning the simultaneous behavior of sodium, potassium, and other ions, in blood stored under various conditions. Unfortunately many of the early studies on red cell permeability reported in the literature were done on red cells devoid of glucose. In the absence of this important substrate the glycolytic system rapidly fails and the escape of potassium and influx of sodium proceed at an excessive rate.

The behavior of potassium in the red blood cell and the plasma under various conditions may be represented diagrammatically as in Fig. 1. In the case of fresh blood collected into

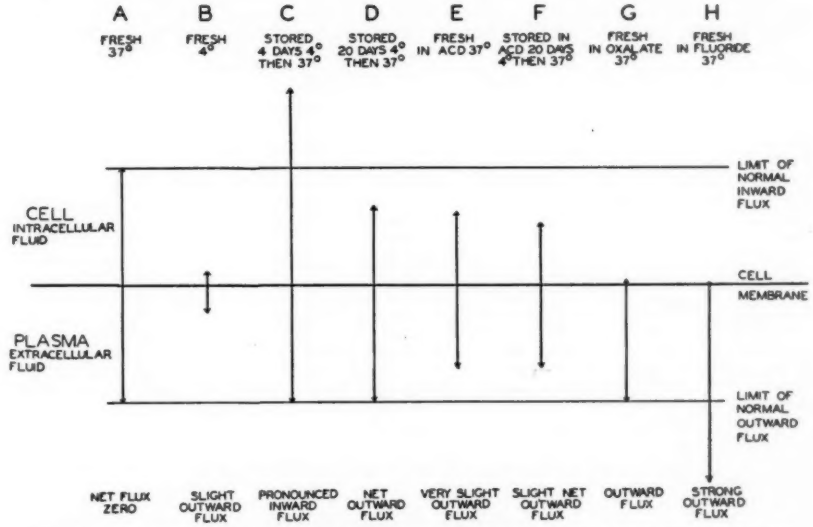

FIG. 1. The movement of potassium ions into and out of the human red blood cell, *in vitro*, under various conditions. The arrows above the central horizontal line represent, in a roughly quantitative way, the movement of potassium ions from the plasma into the cell, while the arrows below the line indicate the simultaneous movement of potassium in the reverse direction.

citrate anticoagulant at 37° C., as represented by the condition *A*, the rate of metabolically driven influx of potassium from the plasma against the high concentration gradient is equal to the rate of efflux, thus giving a constant high concentration of potassium in the cell relative to that in the plasma. If the fresh specimen be placed in the refrigerator (condition *B*), the diminished membrane permeability at the lower temperature decreases the rates of influx and efflux of the ions. However, because of the greatly reduced metabolic activity of the cells the rate of escape of potassium is greater than the rate of gain, resulting in a net loss or outward flux. Condition *C* represents the behavior of a specimen containing citrate and added glucose, and which after storage at 4° C. for four days was returned to a temperature of 37°C. The increase in metabolic activity at the higher temperature is sufficient to effect a regaining of most of the potassium that had escaped during the storage period (and the expulsion of sodium that had been gained). The result is a pronounced net influx of potassium. If the storage period at 4° C. is prolonged to 20 days, the glycolytic activity begins to fail despite the presence of an ample supply of glucose and thus, when the sample is again returned to a temperature of 37° C., the capacity of the cells to regain potassium may not be sufficient to compensate for the normal rate of loss, thus resulting in a net efflux. The latter will become increasingly greater as the glycolytic activity of the cell fails during storage. Condition *E* represents a fresh sample of blood collected in the ACD medium at 37° C. The lowered pH of the plasma (pH 6.8) and consequently of the cells causes a decrease in the rate of utilization of glucose and in the general glycolytic activity. The rates of influx and efflux of potassium thus are considerably reduced and there is a slight net efflux of this ion. Condition *F* refers to the sample when placed at 37° C. after having been stored for 20 days at 4° C. Because of the superior preservation of the glycolytic system in the ACD medium, the capacity to regain potassium is considerably greater than in the corresponding sample preserved in citrate–dextrose for the same period. The glycolytic system, however, fails in these cells also, though at a slower rate.

Condition *G* represents the behavior of blood collected into oxalate solution. This anticoagulant, unlike citrate, enters the cells and inhibits glycolysis, presumably by binding magnesium ions which are essential to the activity of certain enzymes of the glycolytic system. Consequently there is little or no regaining of potassium, and, with a normal rate of loss, the net result is a pronounced efflux. Oxalate can be removed from the cells by washing them with isotonic saline, and the glycolytic activity thereby restored. Storage of blood in oxalate medium, however, leads to an accelerated rate of glycolytic failure. If fluoride is added as anticoagulant, as represented in condition *H*, it not only poisons and inhibits the glycolytic system, but also damages the cell membrane. The result is a greater rate of loss of potassium than results from the inactivation of the glycolytic mechanism alone.

Unfortunately data on the simultaneous behavior of sodium and potassium under all the conditions described above are not available. It is safe to say, however, that the behavior of sodium is generally the reverse of that of potassium and the shift tends to be slightly smaller. In the case of fluoride, iodoacetate, and other glycolytic poisons the equivalence between the movements of sodium and potassium no longer exists since the movements are no longer under metabolic control.

With reference to other ions, such as magnesium and calcium, the former is confined largely to the inside of the cell and the latter to the extracellular fluid. If any exchange of these divalent ions occurs it is probably insignificant. Phosphate is a most important anion which passes freely from the cell to the plasma and in the reverse direction at 37° C. During storage of blood in the cold, the organic phosphate esters tend to undergo hydrolysis, thus liberating inorganic phosphate. Because of the greatly reduced membrane permeability the inorganic phosphate tends to accumulate in the cells. The accumulation has a considerable effect upon the movement of potassium and sodium. When cold-preserved blood is restored to a temperature of 37° C., a resynthesis of the phosphate esters occurs depending on the age of the cells, i.e., on their glycolytic capacity.

The above described behavior is fairly typical of that of all somatic cells under the various conditions. Potassium therapy is sometimes applied in the treatment of infantile diarrhoea and other conditions in which potassium is lost. It is important to appreciate that in a condition of catabolic potassium loss the uptake of potassium by the cells cannot be forced by the simple administration of potassium and indeed, the giving of potassium may produce toxic effects. During recovery, on the other hand, when the cells tend to regain potassium, the administration of these ions, especially in cases of potassium deficiency, may be beneficial.

The movements of ions between the intracellular and extracellular fluids of the structural body tissues are under humoral control. There is much need for quantitative study of the influence of the hormones which control metabolism and other properties of the cells, on the movement of the various anions and cations.

GENERAL DISCUSSION

Dr. J. A. Dauphinee.—I am sure we have all been much interested in the ionic transport mechanism in red cells as outlined by Dr. Denstedt. We must not be tempted, however, to

make too widespread an application of these principles. In some animals, for example dog and cat, sodium is the principle intracellular cation of the erythrocyte and not potassium. Have you carried out, on these "high-sodium" cells, investigations similar to those you describe ?

Dr. O. F. Denstedt.—This question has been put many times to workers who have studied the movement of electrolytes in blood. No one appears to have taken the time to study the simultaneous behavior of sodium, potassium, and other ions in the red cells of species such as the dog and cat, during storage of the blood in the cold. One may predict, however, that during failure of the glycolytic system the ionic gradients between the intra- and extra-cellular fluids will be progressively decreased and ultimately will reach equilibrium. Since the egress of any cation from the cell must be accompanied either by the entry of another or the escape of an anion, it would be interesting to know how the various electrolytes behave in blood in which there is a high gradient of sodium and almost no gradient of potassium.

Dr. A. S. V. Burgen.—I should like to ask Dr. Manery to spend a minute or two on the Gibbs-Donnan equilibrium. Although we all teach students about this, I do not know of any cell system where it is strictly obeyed. Is it because we are dealing with steady states and not with equilibrium ?

Dr. J. F. Manery.—I have not prepared my lecture on the Gibbs-Donnan equilibrium for today. I think you are quite right—we are dealing with steady states in living cells and not with true equilibria. When we realize this, and also the fact that certain assumptions have to be made in order to estimate intracellular ion concentrations, it is amazing to observe how closely the Gibbs-Donnan distribution ratios for the diffusible ions (H^+, Cl^-, and HCO_3^-) approximated each other and a calculated theoretical ratio in red cells (Rapoport, *et al.* (J. Biol. Chem. 131 : 675. 1939; and 163 : 411. 1946) and Bernstein (Science, 120 : 459. 1954)). In soaked frog muscle, too, Boyle and Conway (J. Physiol. 100 : 1. 1941) showed that $[K]_{in} \times [Cl]_{in} = [K]_{out} \times [Cl]_{out}$ and presented these findings to demonstrate that a Gibbs–Donnan equilibrium had been established. The objection has been raised that this muscle was abnormal because the external potassium concentration required to produce the condition was higher than normal. You may say too that the red cell is unlike other cells since its respiration is so low. We must first be able to determine accurately ionic concentrations inside and outside of cells before any of these ideas can be tested.

We must also know whether the movements of ions to which the cell is "permeable" are passive or supported by some sort of metabolic pump, as some suggest exists in muscle for the extrusion of sodium from muscle cells. The distribution of actively transported ions would not be expected to conform to the Gibbs–Donnan distribution laws, but it may influence the movements of passively transported ions and of water. Hence the situation in normal living cells is very complex.

On the other hand, it must be accepted that wherever more non-diffusible ions exist on one side of a membrane than the other, an unequal distribution of the freely diffusible ions will occur. This is clearly exemplified in the distribution of ions across the capillary wall.

THE RELATION OF IONS TO METABOLISM IN BRAIN[1]

By K. A. C. Elliott

I was grateful for the invitation to take part in this symposium because it has forced me to put together a number of observations and to seek hypotheses which would link them together. I must confess that so far I have failed in the effort to find a unified hypothesis although I believe there are sufficient data already available to provide a basis for an inspiration. Perhaps this inspiration will come to those who listen to this summary; perhaps it will come to me as a result of hearing the other speakers.

I shall confine myself largely to results of studies of slices of cerebral cortex *in vitro* and I shall mention results in an order which suits the discussion rather than in the order of their discovery.

Potassium Concentration

Cerebral cortex contains on the average 104 meq. of potassium per kilogram of tissue or 130 meq. per liter of total water. The amounts do not vary obviously from species to species. The sodium content is about 70 meq. per liter of water. It is generally believed that the cells of the brain are in contact with a fluid having about the same electrolyte content as cerebrospinal fluid. This contains on the average about three milliequivalents of potassium and 140 meq. of sodium per liter.

Terner, Eggleston, and Krebs (27) found that retina or brain slices, when suspended under anaerobic conditions in a bicarbonate-buffered Ringer-type medium at 38°, lost potassium to the medium and gained sodium. Under aerobic conditions, when glucose was provided as substrate and particularly when glutamic acid was also present in the medium, the loss of potassium by brain was much decreased. With retina, which had lost some potassium during transport and manipulations, the potassium content would actually increase during aerobic incubation.

Dr. Hanna Pappius and I have confirmed the observations with brain and have extended them as follows. These results must still be regarded as preliminary.

When slices of rat cerebral cortex are incubated *anaerobically* in a glucose-containing, bicarbonate-buffered, saline medium having a composition similar to that of cerebrospinal fluid, they lose potassium very rapidly. Within about 30 min. the concentration of potassium in the slice is nearly as low as in the medium (Fig. 1). *Aerobically* the slices lose about 50% of their potassium in the first 5 or 10 min. Thereafter their potassium content remains constant, or, if glutamate is present, increases considerably though it does not

[1] *Manuscript received December 20, 1954.*
Contribution from The Donner Laboratory of Experimental Neurochemistry, Montreal Neurological Institute, and the Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec. This paper was presented at the Symposium on Electrolytes held as part of the Annual Meeting of the Canadian Physiological Society, Toronto, Ontario, October 22 and 23, 1954.

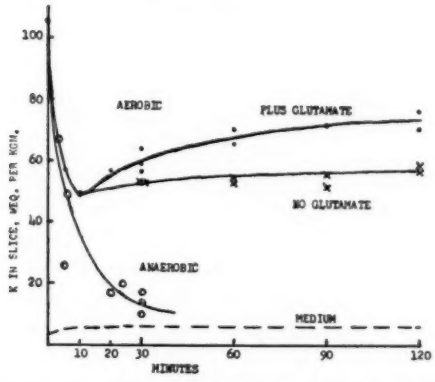

FIG. 1. Changes in tissue potassium concentration during incubation of rat cerebral cortex slices. The amounts of potassium are given as meq. per kgm. of fresh tissue after correcting for swelling on the assumption that the water of swelling has the same ionic content as that of the medium and may be considered part of the medium.

return to the full initial concentration (Fig. 1). Presumably the initial rapid loss of potassium is partly irreversible loss from cells damaged in slicing and partly washing out of potassium which has leaked from more or less intact cells under the adverse conditions during the preparation of the slices. The effect of glutamate is apparently not just to help maintain the tissue potassium but to cause potassium uptake by the tissue.

When slices are incubated anaerobically in media containing varying concentrations of potassium, they lose potassium until the concentration in the water in the slice is only a little higher than in the medium (Fig. 2). The potassium in the slice thus comes into almost free equilibrium with the medium except for a small amount which seems to be somehow bound in the tissue. Aerobically the potassium content of the slice increases with increasing concentration in the medium so that its concentration in the water in the slice is always higher, though decreasingly so, than in the medium (Fig. 2).

If oxygen is supplied after slices have lost most of their potassium as a result of anaerobiosis in a low potassium medium, potassium is reconcentrated in the slices reaching a maximum in about 30 min. The concentration reached is again influenced by the concentration in the medium and is always higher than in the medium (Fig. 3). The recovered potassium level is never quite as high as in tissue which has not been previously subjected to anaerobiosis, presumably because some irreversible damage has occurred.

The maintenance of potassium under aerobic conditions without glutamate seems to be about the same in phosphate-buffered as in bicarbonate-buffered medium but the effect of glutamate in causing a rise of tissue potassium is scarcely apparent when phosphate replaces bicarbonate. Dr. Hugh McLennan informs me that he has found better maintenance of potassium in nerve with bicarbonate than with phosphate-buffered medium. The reason is not known.

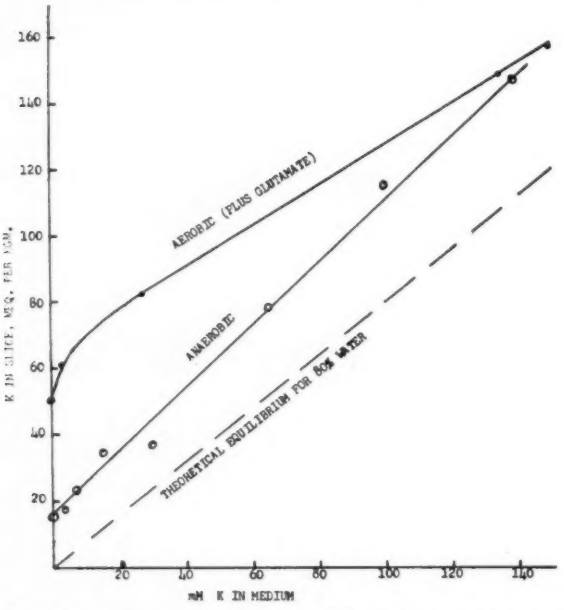

FIG. 2. Effect of the potassium concentration of the medium on the potassium content of brain slices incubated for 60 min.

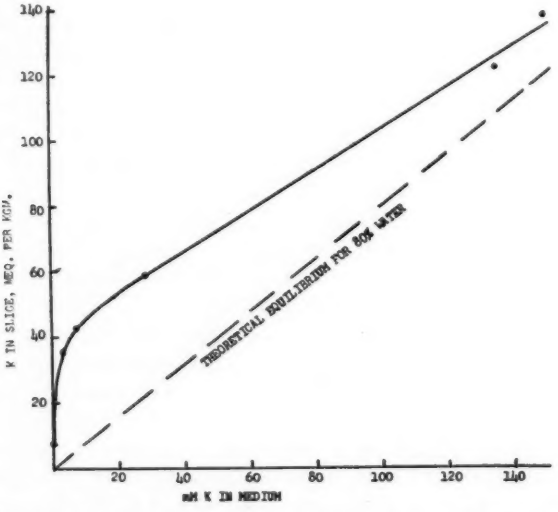

FIG. 3. Effect of the potassium content of the medium on the reconcentration of potassium. Slices, which had lost most of their potassium as a result of 30 min. anaerobic incubation in a low-potassium medium, were incubated aerobically for 60 min. Glutamate, 5 m*M*, present.

Sodium and Potassium and Metabolism

Brain tissue in a Ringer-type medium with glucose respires actively at a constant rate. Contrary to earlier belief, we find that it is capable of active aerobic glycolysis which, however, falls off after a variable, usually short, time. Anaerobically, the tissue shows highly variable but usually continuous, active, glycolysis.

The respiration of a brain suspension in isotonic sucrose–phosphate solution is accelerated by the substitution of various sodium salts for some of the sucrose (10) but whether this is due to the sodium ion, the anions, or to a non-specific electrolyte effect is not clear.

Ashford and Dixon (1), and Dickens and Greville (4) found that high concentrations of potassium, 100 mM, added to an ordinary medium caused a marked, but often short-lived, increase in the respiration of brain slices, a very marked increase in, or maintenance of, aerobic glycolysis and a strong *inhibition* of anaerobic glycolysis. The earlier workers reported that the effects were not obtained if potassium was increased at the expense of sodium in the medium and they added solid potassium chloride rendering the medium hypertonic. Dixon (5), however, has found that the effects of elevated potassium concentration could be obtained in isotonic medium if less potassium, 40 mM, was used so that the sodium concentration could be kept fairly high. There are uncertain points and complicating considerations. Webb (28), for instance, in my laboratory obtained smaller effects than have others, apparently due to unexplained variations in technique. Dickens and Greville reported that brief exposure to a medium completely free of potassium abolished the effect of potassium subsequently added.

Lipsett and Crescitelli (14) found that the stimulation of respiration was abolished by the presence of glutamate or members of the tricarboxylic acid cycle. Perhaps these substances displace glucose or its derivatives as substrate for respiration and are not susceptible to the potassium stimulation. Kimura and Niwa (13) find that the stimulated respiration is inhibited by malonate, which has little effect on the unstimulated respiration. This seems to indicate either that the stimulated metabolism follows a different course from the unstimulated or that potassium affects the permeability to malonate.

The rapid loss of aerobic glycolytic activity in an ordinary medium may be connected with the initial rapid loss of potassium. In a high-potassium medium this loss may be prevented so that the aerobic glycolysis remains high. Weil-Malherbe (29) found that glutamate gradually increases aerobic glycolysis. This may be connected with its effect of helping to maintain or restore the potassium content of the slice.

Anaerobically, glycolysis in an ordinary medium is active in spite of the fact that the tissue potassium is low, and this glycolysis is inhibited if the potassium content is increased by increasing the potassium content of the medium. The low potassium content of a slice under anaerobic conditions implies a high sodium content. Racker and Krimsky (24) found that sodium

strongly inhibits respiration and glycolysis by supplemented extracts, yet a brain slice usually glycolyzes most actively under the conditions in which its sodium content is high.

The Pasteur effect, that is the inhibition of glycolysis by aerobiosis, and the effects of potassium in stimulating aerobic but inhibiting anaerobic glycolysis have not yet been explained. There are other substances, e.g. ammonium, glutamate (29), and veratrum alkaloids and cardiac glycosides (30), which stimulate aerobic and inhibit anaerobic glycolysis.

The effects on metabolism of high potassium undoubtedly reflect physiological properties of the tissue though their significance has not been clearly worked out. It seems that the extra respiratory activity produced by high potassium in the medium corresponds to the extra respiratory activity that accompanies nervous activity. McIlwain (17) and Ghosh and Quastel (12) have shown that this extra respiration is suppressed by narcotics in pharmacologically active concentrations, that is, in lower concentrations than are required to inhibit respiration which has not been stimulated by potassium.

McIlwain (15, 17) has found that application of oscillating electrical potentials to brain slices or fragments increases their respiratory and glycolytic activity. Like the effect of external potassium, this stimulation is inhibited by low concentrations of narcotics. The oscillating potentials may exert their effects via displacements of potassium, sodium, and possibly calcium ions or they may cause a stimulation of neurones. Perhaps these are aspects of the same phenomenon.

McIlwain and his co-workers (16, 18, 19) have also found that high potassium, or glutamate, or oscillating potentials, causes a fall in the phosphocreatine content of incubated slices. This may be a clue to the effects on glycolysis.

Another marked effect of potassium is in the production of acetylcholine. As Quastel and his co-workers (22) found, acetylcholine synthesis by brain slices is stimulated about tenfold by raising the potassium concentration of the medium to about 30 m*M*. Quastel at first thought that this effect was due to potassium acceleration of release of acetylcholine from a bound situation. Later it was shown that potassium is an essential factor in the enzymatic synthetic mechanism. Now it is apparent that a slice incubated even in an ordinary low potassium medium contains a considerable concentration of potassium within itself. It seems as if the effect of elevated potassium in the medium may be exerted on certain cellular surfaces releasing occluded acetylcholine more or less according to Quastel's original hypothesis. Perhaps this kind of action on surfaces might be the cause of some of the other metabolic effects of potassium just as it is concerned in electrophysiological effects on nervous structures. Surface actions of potassium are not too difficult to imagine since Folch has found potassium associated with complex lipids which he has isolated.

Other Cations and Anions

Surface actions of divalent cations are more readily understood and are well known. Calcium exerts a stabilizing effect on brain tissue metabolism as well as on nervous function. In the absence of calcium, respiration is initially more active but falls off more rapidly (4, 10). Magnesium in the medium exerts a similar but less marked effect on whole tissue preparations. Magnesium is, of course, concerned in a great many enzyme-catalyzed reactions.

The effect of lack of calcium may perhaps work through a similar mechanism to that of high potassium. Both cause initial acceleration of respiration followed by a decline in rate. Both also decrease phosphocreatine in slices (18). Possibly the effects of these conditions are due to a kind of stimulation of the nervous structure or at least related to such stimulation. Stimulatory effects of potassium on nervous structures are well known and it is known that lack of calcium, especially if any calcium present is immobilized by citrate, causes nerves and ganglia to show hyperirritability or spontaneous activity and that intracisternal injection of citrate provokes convulsions.

McLennan and I (20) found that for maximal synthesis of acetylcholine by brain slices, the presence of 1.3 m*M* calcium, the concentration in spinal fluid, is essential. Higher and lower concentrations inhibit. Magnesium has similar but smaller effects. It was also found that the bicarbonate – carbon dioxide buffer system is required for maximal synthesis. Any deviation from normal plasma concentrations of carbon dioxide and bicarbonate, even at constant pH, caused a decrease in the amount of acetylcholine produced. No such effects have been reported with cell-free enzyme preparations which synthesize acetylcholine. It therefore seems that calcium and bicarbonate – carbon dioxide serve some function, by surface action or permeability characteristics, whereby tissue structure and the proper co-ordination of some enzyme systems are maintained in the slice.

Mrs. Birmingham and I (2) found that brain tissue in bicarbonate-buffered medium respires slightly less actively than in an unbuffered medium of the same pH. Addition of phosphate to a calcium-free medium usually causes temporary acceleration of respiration followed by decreasing activity. Probably phosphate immobilizes the small amounts of calcium associated with the tissue. Glycolysis occurs at the same rate in phosphate as in bicarbonate-buffered medium.

The effects of hydrogen ion concentration cannot be properly assessed with sliced tissue because the pH of the slice fluids is not the same as that of the suspending medium (7). But with actively respiring suspensions of brain in isotonic medium the respiration shows an optimum at pH 7.0–7.5. Ordinarily anaerobic glycolysis also shows an optimum at about pH 7 but if a trace of pyruvate is added, which stimulates respiration, the rate increases with pH at least up to pH 7.9. Aerobic glycolysis also increases with increasing alkalinity.

The magnitude of the effects of variations in pH or bicarbonate – carbon dioxide concentrations, over the widest physiological or pathological range for plasma, is not great enough to be of much significance on brain metabolism *in vivo* though there may be some regulatory influence of or by glycolysis.

Swelling and Water Distribution

Electrolyte effects have to be considered in relation to movements of water. Some years ago I noted the marked swelling which brain slices undergo when immersed in isotonic solutions (6). This swelling increased when potassium replaced sodium in the medium and decreased greatly when the calcium concentration was high. A curious effect became apparent when it was found that, as judged by the swelling, sucrose or glucose exerted almost no osmotic action on the tissue, unless some electrolyte was present. With a small amount of electrolyte present, sucrose and glucose could prevent swelling or even cause shrinkage.

In conjunction with our work on potassium movements, Dr. Pappius and I are expanding the old preliminary studies on swelling. A slice immersed in a Ringer-type medium at 38° and provided with glucose and oxygen swells rapidly for 15–30 min. and then more slowly. Its weight increases by around 50% or more in an hour in bicarbonate-buffered medium but somewhat less with phosphate buffer (Fig. 4).

Attempts to determine the nature of the swelling have produced puzzling results. In a study of the swelling of kidney slices, Robinson (25) concluded that, if the inulin space is taken to represent extracellular space, then the intra- and extra-cellular spaces swelled in equal proportions. Calculation

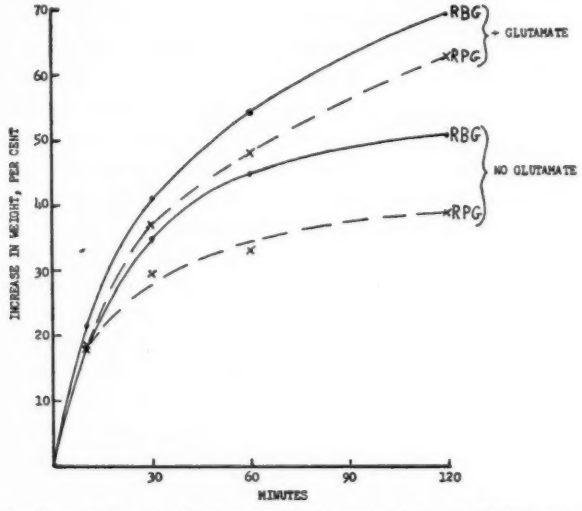

FIG. 4. Swelling of incubated slices in bicarbonate-buffered (RBG) and phosphate-buffered (RPG) glucose-containing Ringer-type medium with and without glutamate, 5 m*M*.

(see (9)) from his water determinations show, however, that his kidney slices swelled only about eight per cent under the conditions allowing about 50% swelling of brain slices.

We find that inulin seems to occupy a fairly constant percentage, about 30% of the total swollen slice, within 30 to 60 min. But at this time, when the tissue has swollen, one would expect the extracellular water to be about 65% of the total swollen slice if we assume that initially 30% of the slice volume was extracellular and that all the swelling was extracellular. This discrepancy would lead to the conclusion that most of the water of swelling is intracellular or otherwise not occupied by inulin.

Thiocyanate, however, gives a different picture. It is very rapidly taken up by the tissue and gives, within five minutes, a thiocyanate space which equals about 65% of the total swollen volume. The extracellular space of whole brain is often stated, on the basis of sodium and chloride spaces, assuming these ions to be extracellular, to be 30 to 40% of its volume. Our sodium determinations on rat cerebral cortex (23) indicate a sodium space of 35–45%. It is likely that our slices, in which cells have been damaged by slicing, would have an elevated, effective, initial, extracellular space. On the assumption that the initial extracellular space of the slice is about 45%, the swelling seems to be almost all thiocyanate space or extracellular.

Sucrose, like inulin, is taken up more slowly than thiocyanate, but eventually it seems to occupy the same space as thiocyanate, that is, greater than inulin. Thus thiocyanate and sucrose seem to occupy a space which is fairly well defined but greater than that occupied by inulin (Fig. 5). It seems as if there are three spaces—the fully extracellular, an intermediate, and an intracellular. Thiocyanate and sucrose, but not inulin, can occupy the intermediate space. The fact that thiocyanate and sucrose occupy the same space seems to indicate that molecular size rather than charge is the factor determining the space occupied. It seems possible that some structure in the tissue hinders the large inulin molecule from entering all the actual extracellular space. The swelling seems to take in the part of the extracellular space which is not accessible to inulin.

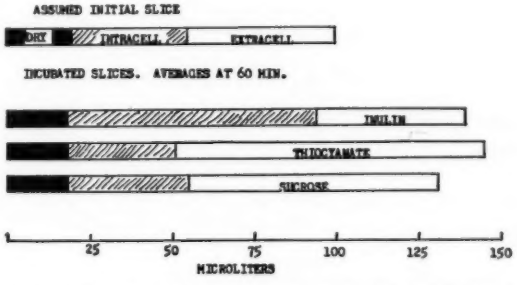

FIG. 5. Inulin, thiocyanate, and sucrose spaces of incubated brain slices. Total lengths represent weight after incubation per 100 mgm. of initial weight. Black portions represent dry weight. Open portions show volume occupied by inulin, thiocyanate, or sucrose. Shaded portion, obtained by difference, represents apparent intracellular space.

The addition of glutamate to the medium has interesting effects. It distinctly and consistently increases the degree of swelling (Fig. 4) and this swelling is accounted for by increase in the apparent *intracellular* space. Determinations of thiocyanate, sucrose, or inulin space all indicate this (Fig. 6).

It seems that the effect of glutamate in increasing potassium concentration can be accounted for by this increase in intracellular space. The average of the thiocyanate and sucrose spaces indicates that the intracellular space in a slice incubated without glutamate is about 36 μliters per 100 mgm. of original weight. The potassium found (about 57 meq.), if all in this space, would have a concentration of about 158 mM. When glutamate is present the potassium content is increased (to about 75 meq.) but the intracellular space is increased to 51 μliters which would bring the potassium concentration in this space to 147 mM. Thus, the potassium *concentration* has not actually increased. It is possible that a similar consideration may be applied to the finding of Stern, Eggleston, Hems, and Krebs (26) that glutamate is concentrated in incubated brain slices. The increase in tissue concentration which they obtained was large but the initial value was much lower than in intact brain, probably because of loss by metabolism during preparation of the tissue. Their increase could have been partly a restoration of the normal concentration and partly due to increase in the intracellular space.

When slices are incubated in a high potassium medium, the swelling and intracellular space are increased and the presence of glutamate has no effect. Thus potassium and glutamate act in the same way and their effects are not additive.

We now see that potassium and glutamate are both actively concentrated in the tissue and have similar effects on aerobic and anaerobic glycolysis, on the phosphocreatine content of the slice, and on swelling and intracellular space.

With kidney cortex slices, Robinson (25) observed clear correlations between energy metabolism and suppression of swelling. So far our results do not seem to show any marked correlation between energy metabolism, potassium

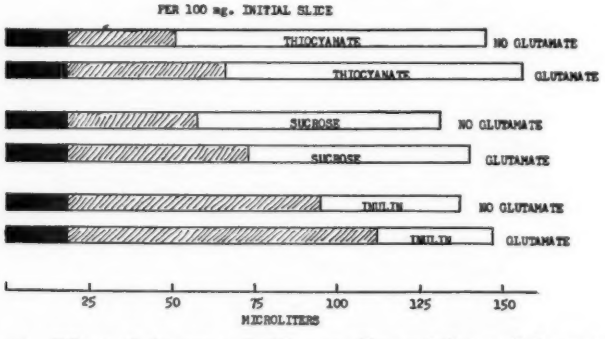

FIG. 6. Effects of glutamate, 5mM, on swelling and "intracellular space."

movements, and swelling in brain slices. Swelling occurs anaerobically when there is no respiration and the tissue potassium has greatly decreased. But nearly as much swelling also occurs, increasing with time, aerobically, though respiration is going on actively and at constant rate, and the tissue potassium content is fairly maintained or increasing. The presence of glutamate, in addition to glucose, aids the uptake of potassium by the slice and either has no effect on, or slightly increases, the respiration rate but it increases the extent of swelling. High potassium accelerates aerobic metabolism but increases swelling

The figures given earlier for tissue potassium concentration are based on the fresh weight of tissue and on the assumption that the water of swelling is all extracellular and can be considered to be part of the medium and have the same potassium concentration as the medium. This is the assumption made by Terner, Eggleston, and Krebs (27). The experiments with inulin, thiocyanate, and sucrose were done at first merely to check this. The thiocyanate and sucrose experiments suggested that the assumption was more or less valid. But attempts to avoid the correction for swelling were made by applying the earlier observation that sucrose, in the presence of electrolyte, exerts osmotic effects on brain slices. In a medium containing the normal electrolyte concentrations and, in addition, 10% sucrose, almost no swelling occurred. However, the thiocyanate space was relatively greatly increased, becoming 72% of the tissue (Fig. 7). The osmotic effect of sucrose evidently removed a large proportion of the intracellular water. As would be expected, there was an accompanying loss of potassium but less than would be expected from the decrease in intracellular space. There was also a decrease in respiration rate.

The swelling of tissues and of mitochondria in isotonic media has led various authors to conclude that the intracellular fluid is hypertonic compared to plasma, and that water is actively extruded. Robinson concluded that its osmolarity is between 50 and 100% greater than that of extracellular fluids. Our results with 10% sucrose in saline medium, which is twice isotonic, indicate that intracellular fluid in cerebral cortex is certainly less than 100% hypertonic. In earlier experiments (6) it was found that swelling occurred

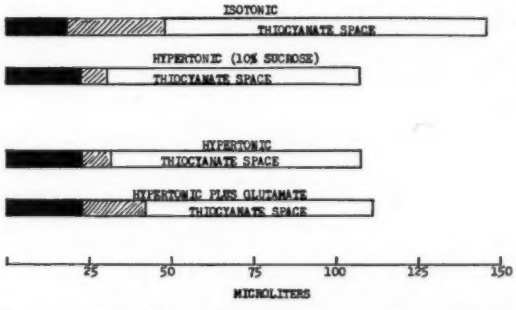

FIG. 7. Effects of adding 10% sucrose to the usual incubation medium rendering it twice isotonic.

even in four times isotonic saline, though not in saline–sucrose mixtures. This is evidently due to permeability of the cells to chloride and to some extent to sodium, though there may be changes in state of tissue constituents *in vitro* which allow them to take up more bound water. Under normal conditions *in vivo* the tendency to swell may be controlled by hormonal or other factors.

Unfractionated brain suspensions prepared in isotonic saline medium show remarkably well preserved respiratory and glycolytic activity and Pasteur effect (7, 8, 10, 11). In these unfractionated suspensions cell structure must be pretty well disrupted but the subcellular particles nevertheless retain much of their activity in isotonic medium. Suspensions prepared in hypotonic medium are much less active, and irreversibly so, presumably because the organized enzyme complexes (mitochondria) are disrupted by hypotonic media. (Not all chemical functions are preserved in normal condition in unfractionated isotonic suspensions—acetylcholine synthesis for instance cannot be increased by potassium probably because of destruction of the necessary adenosine-triphosphate (21) by the ATPase released on disruption of the tissue.)

I have no need to elaborate on the known exchanges of electrolytes which occur during normal and heightened electrical activity in nerve and muscle. There have been few direct observations on brain *in vivo*. Colfer and Essex (3) showed, by microincineration methods, that, during convulsions in experimental animals, there is a shift of potassium from the inside to the outside of neurones in the brain and a converse shift of sodium. This was to be expected. We have been unable to detect any chronic change in the sodium and potassium contents of human focal epileptogenic cerebral cortex (23).

Changes in the water content and volume of the brain *in vivo* can be produced by hypo- and hyper-tonic infusions (e.g. (9)) but the osmotic pressure of body fluids are not likely to show spontaneous changes large enough to produce such edema. It seems likely that that cerebral edema, when it occurs, may be due to changes in some regulatory factors so that the same process occurs as in a slice immersed in isotonic saline medium.

I have not attempted to mention all the many detailed observations on movements of ions and on the effects of composition of media on brain metabolism but I believe I have said enough to indicate that some enlightened experimentation and thinking along the lines I have discussed, together with those discussed by others in this symposium, should help the solution of various problems.

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DISCUSSION: R. J. Rossiter[1]

In the preceding paper Dr. Elliott (5) has given a comprehensive review of the effect of electrolytes on the metabolism of respiring brain slices and the movements of water and certain inorganic ions. In particular, he stressed the effects of increasing the concentration of K^+ in the suspending medium. He also noted the puzzling similarity between the effects of excess K^+ and those that follow the addition of L-glutamic acid.

Miss Findlay and Mr. Magee in our laboratory have been investigating the effects of electrolytes and glutamic acid on phosphorylating systems in respiring slices of cat and guinea pig brain (6). They have studied the incorporation of inorganic phosphate labelled with P^{32} into the lipid phosphorus, ribonucleic acid phosphorus (RNA nucleotides separated by electrophoresis on paper strips), and two fractions, called for the time being residue organic phosphorus (ROP) and "phosphoprotein" phosphorus (PP), respectively. The techniques employed have been described in previous papers (3, 7, 8, 24). The ROP represents non-nucleotide phosphorus and the recent work of Mr. Middleton shows that this fraction is comprised of seven or more metabolically active phosphorus-containing components, at least two of which contain inositol. The PP yields inorganic phosphate on mild alkaline hydrolysis and may represent the phosphorus of enzyme–phosphate complexes (8).

Table I shows that for slices of cat brain respiring in Krebs Ringer bicarbonate medium, an increase in the concentration of K^+ causes a decrease in the incorporation of P^{32} into each of the four fractions. Omitting Ca^{++} from the medium causes a similar decrease, although to a slightly less degree. This finding is remarkable, since either the addition of K^+ (1,4) or the removal of Ca^{++} (2, 4) causes an increase in the oxygen consumption of brain slices. The addition of NH_4^+ was also shown to cause a similar inhibition of the incorporation of P^{32} into all four fractions (6).

TABLE I

Effect of K^+ and Ca^{++} on the specific activity of lipid P, RNA, ROP, and PP of slices of cat brain incubated in medium containing P^{32}

K^+ (m*M*)	Ca^{++} (m*M*)	Specific activity (counts/min./μgm.P)			
		Lipid P	RNA	ROP	PP
5.9	3.6	15.9	27.2	432	1390
124	3.6	7.1	7.8	95	680
5.9	0	11.5	19.4	107	570

[1] *Contribution from the Department of Biochemistry, University of Western Ontario, London, Ontario.*

TABLE II

EFFECT OF GLUCOSE AND L-GLUTAMIC ACID ON THE SPECIFIC ACTIVITY OF LIPID P, RNA, ROP, AND PP OF SLICES OF CAT BRAIN INCUBATED IN MEDIUM CONTAINING P^{32}

Medium	Specific activity (counts/min./μgm.P)			
	Lipid P	RNA	ROP	PP
No substrate	7.7	—	103	670
Glucose 0.011 *M*	22.7	26.0	305	1360
Glutamate 0.01 *M*	9.4	17.1	167	490
Glucose 0.011 *M* and glutamate 0.01 *M*	14.4	20.1	156	730

TABLE III

SIMILARITY BETWEEN EFFECTS OF K^+ AND GLUTAMIC ACID ON RESPIRING BRAIN SLICES

Effect	References	
	K^+	Glutamic acid
1. Increase in oxygen consumption	(1, 4, 9, 14)	(13, 22, 26)
2. Increase in aerobic glycolysis	(1, 4, 9)	(27)
3. Decrease in anaerobic glycolysis	(1, 4)	(27)
4. Decrease in incorporation of P^{32} into lipid P, RNA, ROP, and PP	(6)	(6, 7, 8, 24)
5. Increase in concentration of inorganic P	(9, 18)	(17, 18)
6. Decrease in concentration of creatine phosphate	(9, 18)	(17, 18)
7. Increase in total intracellular K^+	(5)	(5, 25)
8. Increase in volume of intracellular fluid space	(5)	(5)
9. Abolition of the metabolic response to *in vitro* stimulation	(9)	(17)[1]

[1] *The species studied is of some importance. McIlwain (19) reported that slices of human brain, in contrast to the findings for laboratory animals, demonstrated the usual metabolic response to in vitro stimulation in the presence of glutamic acid.*

Table II shows that not only is L-glutamic acid unable to support the incorporation of the P^{32} into all four fractions, but that it also inhibits the incorporation normally observed in the presence of glucose. Again this finding is of interest, since it is well known that glutamic acid increases the oxygen consumption of brain slices (13, 22, 26). Other experiments have shown that L-glutamine causes a similar decrease in P^{32} incorporation (6).

Dr. Elliott (5) has already commented upon the similarity between the effects of K^+ and those of glutamic acid in increasing the total intracellular K^+ and the volume of the intracellular space of brain slices. Our experiments show that there is also a similarity between K^+ and glutamic acid in their ability to inhibit phosphorylation reactions, without interfering with oxygen consumption. These two substances have similar effects on a number of cellular processes, including the abolition of the metabolic response to *in vitro* stimulation, first described by McIlwain (15, 16, 20) for brain slices respiring in glucose saline. These similarities in respiring brain slices are summarized in Table III. In some instances, the effects are not additive, i.e. the effect of K^+ is not observed in the presence of glutamic acid and *vice versa*. Thus in the presence of glutamic acid an increase in the concentration of K^+ does not affect the oxygen consumption (14) or the volume of the intracellular fluid space of the slice (5).

Similar metabolic effects in brain slices are produced by the addition of NH_4^+ (6, 9, 27), or the removal of Ca^{++} (2, 4, 6, 9, 18) from the medium, by the addition of glutamine (6, 17, 18, 27), by the addition of nitrophenols (4, 7, 8, 18, 20, 21, 24), by the addition of many of the intermediates of the tricarboxylic acid cycle (7, 8, 11, 14, 24), and by the *in vitro* stimulation of the slices (10, 15, 16, 20). Usually the effects are not additive with either the K^+ effect or the glutamic acid effect. For example, an increase in the concentration of K^+ does not stimulate the oxygen consumption of brain slices in the presence of dinitrophenol, or in the presence of many of the intermediates of the tricarboxylic acid cycle (14). It has been seen already (Table III) that the metabolic effect of *in vitro* stimulation is abolished in the presence of either excess K^+ (9) or glutamic acid* (17).

* *The species studied is of some importance. McIlwain (19) reported that slices of human brain, in contrast to the findings for laboratory animals, demonstrated the usual metabolic response to in vitro stimulation in the presence of glutamic acid.*

Some hint as to a possible common factor in this wide variety of conditions is to be found in the observation of McIlwain and colleagues that they are all associated with a decrease in the concentration of creatine phosphate. Also the work in our laboratory indicates that under these same conditions there is a decrease in phosphorylation. These two observations, well documented in the case of excess K^+ or glutamic acid, suggest that the slices are depleted of their stores of energy-rich phosphate compounds. The recent work of McIlwain and his group indicate that not only is there a depletion of creatine phosphate, but also a depletion of ATP (12, 18). This depletion of energy donors is manifested by the inability of the slice to respond to the *in vitro* stimulation.

The mechanism whereby an increase in the concentration of K^+ in the medium can cause a depletion of ATP is not apparent. If it is assumed that the main metabolic processes of the brain cell are directed towards the maintenance of a constant *gradient* between the intracellular and extracellular concentration of K^+, an increase in the concentration of K^+ in the medium, by diminishing this gradient, would cause an increase in the energy demands of the cell. Presumably the process whereby K^+ is concentrated is similar to that described by Terner, Eggleston, and Krebs (25). Glutamic acid, which is itself concentrated in brain slices (23), is also needed for the maintenance of the K^+ gradient. It may thus be that either a high concentration of K^+ in the suspending medium (decrease in K^+ gradient), or the addition of glutamic acid, initiates a metabolic response that is similar in each case. This response is accompanied by the movement of K^+ into the cell against a concentration gradient. Such a movement of K^+, an energy-requiring process, could cause a depletion of ATP stores.

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GENERAL DISCUSSION

Dr. K. C. Fisher.—I should like to ask Dr. Elliott what he thinks of the possibility that, in work of this kind where citrate or related compounds are used, the observed effect might be due to lack of ionized calcium and not to citrate per se. We have demonstrated that the so-called citrate effect on the respiration of frog muscle could be attributed entirely to a low concentration of calcium ions.

You describe experiments in which brain slices take up potassium in the presence of glutamate. One of the effects of glutamate, then, is to reduce the external potassium concentration and destroy the ionic balance of the physiological salt solution. Lowering the potassium concentration outside might be expected to have the same effect as raising the concentration of calcium ions. Do you know whether or not the relative concentration of the calcium ion is of any significance in this connection?

Dr. K. A. C. Elliott.—One would have to know in each specific case whether the effect of citrate was caused by reduction of the calcium ion concentration or could be attributed to its function as an enzyme substrate. Intracisternal injections of citrate are believed to cause convulsions by reducing the calcium ion concentration.

I am confused about glutamate. I feel that its function here may not be the usual one of a metabolite. It can stimulate respiration though it is not very effective in the presence of glucose. It may act merely as another intracellular anion. Suppose glutamate cannot normally permeate brain cells whereas glutamine can; the latter could be produced from glutamate and, after entering the cell, be hydrolyzed and converted to glutamic acid increasing the intracellular anion content and therefore the intracellular cation content. High potassium and glutamate inside the cells would cause a rise in intracellular osmotic pressure with the resultant swelling of cells.

Dr. J. J. Ghosh.—I would like to ask Dr. Rossiter whether this stimulatory effect of excess potassium on brain slice respiration has any similarity to the "uncoupling" effect of 2,4-dinitrophenol ?

Dr. R. J. Rossiter.—To show the "uncoupling" effect of dinitrophenol, homogenate or mitochondrial preparations rather than preparations containing whole cells have been employed. In such cell-free systems, potassium stimulates both oxygen consumption and phosphorylation, whereas dinitrophenol stimulates oxygen consumption but inhibits phosphorylation. In systems with intact cells (for example slice preparations), dinitrophenol also stimulates oxygen consumption and inhibits phosphorylation. In brain slices, but not in slices from other tissues, potassium has an effect on oxygen consumption and phosphorylation similar to that produced by dinitrophenoi. This is in contrast to the effect of potassium on homogenate or mitochondrial preparations, either from brain or other tissues.

Dr. J. J. Ghosh.—Of course, in other systems, like minced rat muscle, Boyer, Lardy, and Phillips (J. Biol. Chem. 149 : 529–541. 1943) have shown that potassium ion has some positive influence on the phosphorylation of the adenylic acid system. The more recent studies with mitochondrial and microsomal preparations also support this view (Pressman and Lardy (J. Biol. Chem. 197 : 547–556. 1952)).

Dr. J. M. R. Beveridge.—Certain differences in electrolyte metabolism in brain tissue compared with other tissues have been presented. Is there any possibility that these differences could be associated in any way with the fact that brain tissue differs from other body tissues in as much as it does not require insulin for the normal or ready utilization of glucose ? This process, in these other tissues at least, is well known to be associated with certain electrolyte shifts between the extra- and intra-cellular fluid compartments, a fact that is of some interest in connection with this presentation.

Dr. K. A. C. Elliott.—I should say quite definitely that insulin does not have a thing to do directly with metabolism in the brain.

Dr. J. M. R. Beveridge.—I did not mean that insulin affected the metabolism of brain.

Dr. K. A. C. Elliott.—You do not find any difference between insulinized and non-insulinized animals with regard to ability of their brains to utilize glucose if it is provided. The respiration of tissue from insulinized animals falls more rapidly if no glucose is provided since it contains less lactate (derived from residual glucose in the tissue). I would not expect electrolyte shifts associated with glucose utilization to be different in insulinized animals. I may say that I do not think glucose goes into cells as such. It probably has to be phosphorylated at the cell surface.

GLANDULAR SECRETION OF ELECTROLYTES[1]

By J. A. Hildes

Introduction

The elaboration of distinctive electrolyte patterns in glandular secretion has been a subject of considerable interest for many years. Although present day interests in this subject are mainly concerned with the intimate cellular mechanisms by which such specific secretions are elaborated, there is still incomplete information on the detailed electrolyte composition of the secretion of various glands, particularly in the human subject.

It is this aspect which I wish to discuss today. Many of the data I shall present have been taken from studies carried out with the collaboration of Dr. M. H. Ferguson of the Department of Physiology and Medical Research, University of Manitoba.

The glands of external secretion fall into two general categories: those whose secretions are isotonic with the blood such as the gastric glands and the pancreas, and those with secretions of variable tonicity such as the salivary glands and sweat glands.

Investigation of the gastric glands, particularly in humans, is complicated by our inability to collect the total output of one type of gland uncontaminated by other secretions so that its rate of secretion can be measured and the juice analyzed. Using animals with isolated gastric pouches does not entirely solve the problem as pure gastric juice is itself considered by Hollander (20) to be a mixture of two or more secretions, each derived from distinct types of cells in the gastric glands—the parietal juice or acid component and the non-parietal juice or alkaline component. Both components are considered to be isotonic or nearly so, with blood. The variations in the electrolyte composition of mixed gastric juice are explained by a varying mixture of these components.

For the above reasons the pancreas has been selected to illustrate the type of gland producing a secretion isotonic with blood. Similarly the salivary glands, particularly the parotids, are more suited than the sweat glands to illustrate hypotonic secreting glands. The total output of each parotid gland can be collected with a small suction cup held in place over the parotid papilla.

The Pancreatic Secretion of Electrolytes

The pancreatic secretion of electrolytes has been the subject of a good deal of experimental work, mainly in dogs. DeZilwa in 1904 (10) compared the freezing points of pancreatic juice and plasma and considered the two fluids to be isotonic. This work has been confirmed and extended by several investigators since then (3, 4, 5, 13, 21, 23, 26).

[1] *Manuscript received December 20, 1954.*

Contribution from the Department of Physiology and Medical Research, University of Manitoba, Winnipeg, Canada. This paper was presented at the Symposium on Electrolytes held as part of the Annual Meeting of the Canadian Physiological Society, Toronto, Ontario, October 22 and 23, 1954.

The concentrations of the major cations in pancreatic juice, sodium and potassium, remain constant or nearly so, in all animal experiments and are very close to plasma levels. The dependence of sodium and potassium concentration on blood levels is further shown by experiments of Ball (5) in which intravascular infusions of sodium and potassium salts were followed by increase in pancreatic juice concentration which closely paralleled the change in serum concentration. These experiments also showed that the sodium and potassium levels were independent of each other (5, 32). The source of the sodium and potassium of pancreatic juice is undoubtedly the blood. Montgomery *et al.* (29) found that intravenously administered labelled sodium rapidly appeared in the pancreatic juice. A relatively slight change in the glandular content of sodium and potassium was found by Oldfelt (30) after prolonged intensive pancreatic secretion.

The anionic composition of pancreatic juice has been the subject of study by several investigators using either anesthetized animals for acute experiments or animals with external pancreatic fistulae (5, 12, 23, 35). Hart and Thomas (16) used unanesthetized animals in which the pancreas was functionally intact and emptying normally into the gut but in which the total pancreatic output could be anaerobically collected at will. All these studies were in general agreement that there was a reciprocal relationship between the two main anions, bicarbonate and chloride, and that the sum of their concentrations was approximately isotonic with blood. The concentration of bicarbonate and chloride appears to depend on the rate of secretion between certain limits but is entirely independent of the type of stimulus used (16). Hart and Thomas (16) found the bicarbonate concentration to be 34 meq./liter at a secretion rate of 0.003 ml./min./kgm. body weight rising to 135 meq./liter as the secretion rate rose to 0.05 ml./min./kgm. Above this secretion rate bicarbonate and chloride concentrations were no longer affected by the rate of secretion. They also found that juice carefully collected under oil had a pH of 8.0 at low rates of secretion and that the pH rose to 8.3 at rates of approximately 0.05 ml./min./kgm. but was not further changed at higher secretion rates.

With regard to human studies, Lagerlof (24) reported that bicarbonate content increased with increasing secretion rate in a subject stimulated with an injection of secretin and that this was associated with a reciprocal fall in the pancreatic juice chloride. It is difficult to be certain of the secretory rates or the concentrations in such an intact subject when collections are made through a duodenal tube. The collections may be incomplete and certainly there was a considerable admixture of bile, the release of which into the duodenum was also stimulated by the secretin used.

There are isolated reports of human pancreatic fistulae in the literature. One of these (19) is shown in the first figure. This subject with a total pancreatic fistula not communicating with the duodenum or bile ducts was subjected to periods of starvation and feeding while the pancreatic juice was collected and analyzed.

The sodium and potassium concentrations in the pancreatic juice were identical with serum levels, shown by closed circles and crosses respectively. This relationship was not influenced by conditions of diet, starvation, or dehydration, nor by the composition of administered parenteral fluids. Nor was it influenced by the administraion of 400 mgm. of Banthine per day given for the duration of the starvation period and for six days during the second control period. It was also quite independent of the daily volume of pancreatic juice.

The chloride concentration on the other hand was constantly lower than the serum level. It was difficult to collect juice from this patient without

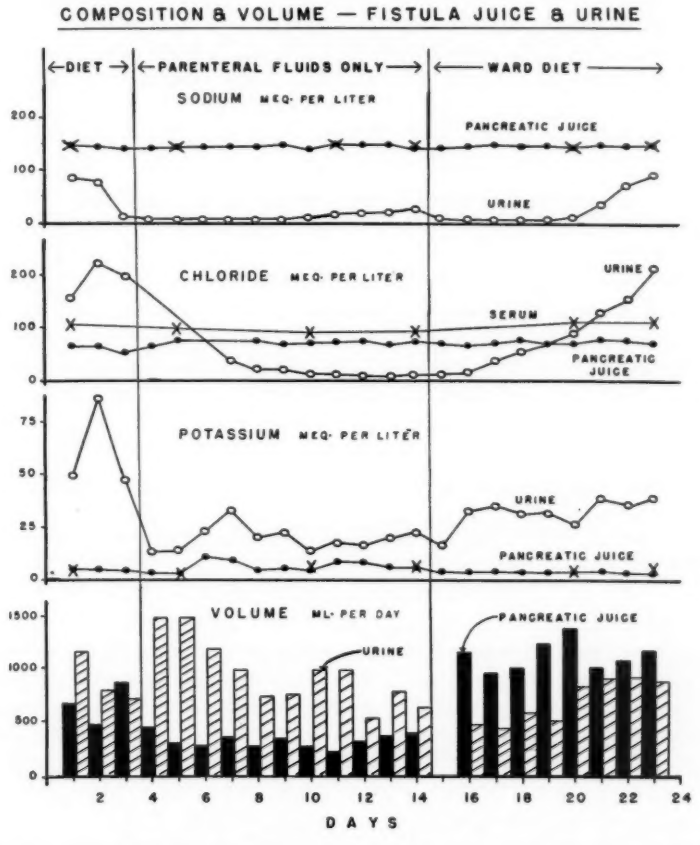

FIG. 1. The relationship between serum (crosses) and pancreatic juice (closed circles) concentrations of sodium, chloride, and potassium in a patient with a total pancreatic fistula. Urine concentrations (open circles) are shown for comparison. Daily volumes of pancreatic juice and urine are shown in the bottom panel. A period of alimentary starvation was preceded and followed by control periods on ward diet. (From Gastroenterology, 21 : 64. 1952.)

exposure to air so that bicarbonate estimation was only available on one occasion during the period of alimentary starvation. It was found to be 53 meq./liter compared to a simultaneous plasma bicarbonate content of 29 meq./liter. This shows the same reversal of bicarbonate and chloride relative to blood levels observed in the numerous animal experiments cited above.

The one major discrepancy between these results and those reported in experimental animals is the apparent independence in this case of the chloride concentration on secretion rate. Two factors may have some bearing on this apparent discrepancy:

(1) Changes in chloride concentration (and reciprocally the bicarbonate) may be sensitive to changes in secretory rate but these could have been masked by the measurement of pancreatic output over 24-hr. periods. Only crude mean rates of secretion can be calculated from these types of data.

(2) The highest mean rate achieved in this case was approximately 1 ml./min. When this is reduced to the common denominator of ml./min./kgm. body weight, the rates of secretion ranged from 0.003 to 0.0175 ml./min./kgm. Although this represents a sixfold increase, the maximum bicarbonate concentration in dogs was not achieved until the gland had been stimulated to secrete at 0.05 ml./min./kgm. (16).

There has been considerable discussion in the literature concerning the source of pancreatic bicarbonate. This has been reviewed by Solomon (32) and will not be elaborated here except to say that there are several unsettled problems (6, 8, 33), including the effect of carbonic anhydrase inhibitors on composition of pancreatic juice.

The Secretion of Electrolytes by the Salivary Glands

In contrast to pancreatic juice, the electrolyte concentration of saliva is variable and appears to be independent of blood levels. However, there is a general pattern of electrolyte secretion by the salivary glands (18). This is shown in Fig. 2.

This illustrates the relationship between the parotid juice concentrations of sodium, potassium, chloride, bicarbonate, and inorganic phosphate, and the rate of secretion found in three normal human subjects who are distinguished by the different symbols.

The juice is always hypotonic but variably so, having a higher electrolyte content at high secretion rates. This makes for less osmotic work at the higher rates of flow. The increasing inorganic content of saliva at high secretion rates was known to Heidenhain (17) as early at 1868. The findings shown here are in general agreement with the reports of Gregersen and Ingalls (15) in animals and with the findings of Thaysen and his colleagues (34) in humans stimulated by an injection of mecholyl.

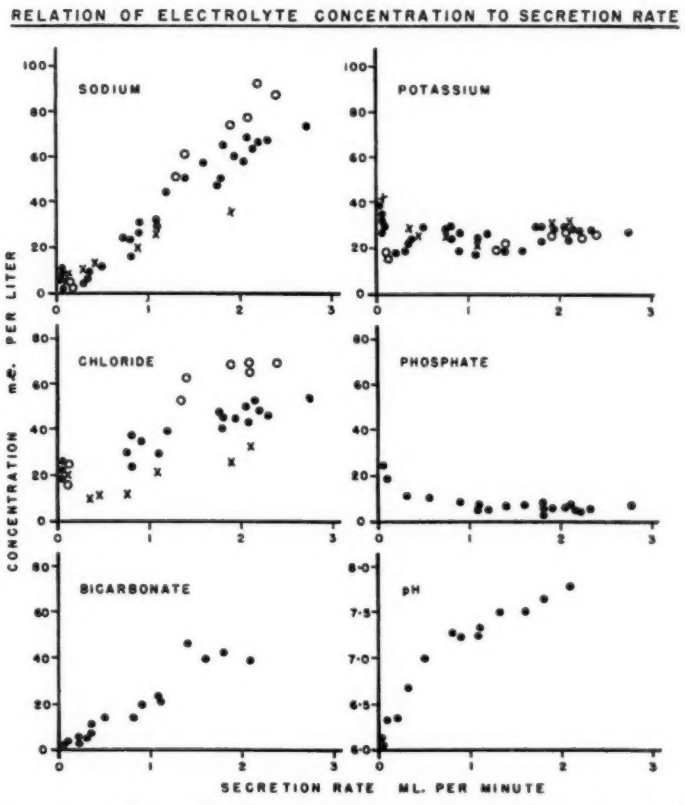

FIG. 2. The relationship between parotid juice concentrations of sodium, chloride, bicarbonate, potassium, phosphate, and the rate of secretion. Data from three subjects with respect to sodium, chloride, and potassium are shown. Samples for the estimation of pH and bicarbonate content were collected under oil.

The sodium concentration starts off at 4–5 meq./liter in the resting juice and rises to 80–90 meq./liter at a rate of 3 ml. per minute but does not reach blood level.

Potassium concentration is relatively constant but it too is not tied to the blood level, being four to five times higher than the serum concentration.

In contrast to the reciprocal relation found between bicarbonate and chloride in the pancreatic juice, in saliva both these ions increase in concentration with increasing secretion rates. The chloride concentration does not reach blood levels but the bicarbonate concentration equals blood levels at approximately 1 ml./min. and rises above this at higher rates. Phosphate concentration is also independent of secretion rate but again in contrast to pancreatic juice it is one and one-half to two times greater than blood level whereas in pancreatic juice it has been found to be one-quarter to one-third of the blood level (5).

Parotid saliva collected carefully under oil has a pH rising from 6.0 to 7.8 as the secretion rate increases from resting levels to 2.2 ml./min. This is a greater change than that found by Hart and Thomas (16) in pancreatic juice. In other words, the parotid juice appears to be not so well buffered.

A phenomenon noted by DeBeer and Wilson (9), by Langstroth, McRae, and Stavraky (25), and by Wills (38) is also apparent in this slide, that is the high concentrations of potassium and phosphate at secretion rates below 0.5 ml./min. This has been attributed by Babkin (2) to water reabsorption from the ducts.

In parotid saliva there is also a fairly wide variation between individual subjects. This can be seen in the sodium and chloride charts (Fig. 1). In studies on other subjects not shown here we found the calculated regression lines for a number of normal subjects to be significantly different one from another. Indeed, there are small but appreciable differences in the same subject tested on different days.

The same is true for the potassium concentration when the mean potassium concentrations at secretion rates greater than 0.5 ml. per minute are compared. A significant difference is noted between subjects and in the same subjects with repeated tests at weekly intervals (18).

In the parotid data I have just shown the stimulus used was graded strengths of dilute acetic acid administered to the midline of the tongue. We have also used glucose candy held in the mouth as another stimulus to see if different stimuli produce quantitative or qualitative differences in the resulting secretion. We could find no differences between the two stimuli that could not be explained by the other variables mentioned (18). Similarly Gregersen and Ingalls (15) found that the secretion of salivary electrolytes in animals stimulated by nerve stimulation and by pilocarpine injection were the same. Wills (38), however, found slight differences between these two stimuli. Subcutaneous mecholyl in human subjects (34) also produced a similar pattern of secretion.

We have also tried to analyze the electrolyte secretion of submaxillary and sublingual glands in human subjects (18). It is difficult to compare these glands with the parotid glands as we have no satisfactory method for collecting the juice of a single gland uncontaminated by other secretions. However, again the same pattern holds with regard to sodium, chloride, and potassium in that the first two increased with rate of secretion whereas the latter was constant; but it is difficult to quantitatively compare the sodium secretion of these glands with parotid juice. With regard to potassium, where the concentration is not influenced by rate of secretion, a comparison is possible and this is illustrated in Fig. 3. The potassium concentration of parotid juice is compared with the potassium concentration in mixed submaxillary and sublingual saliva. The concentration in both cases is constant and independent of secretion rates over 0.5 ml./min. but the level in the parotid saliva was appreciably higher than that in the mixed saliva.

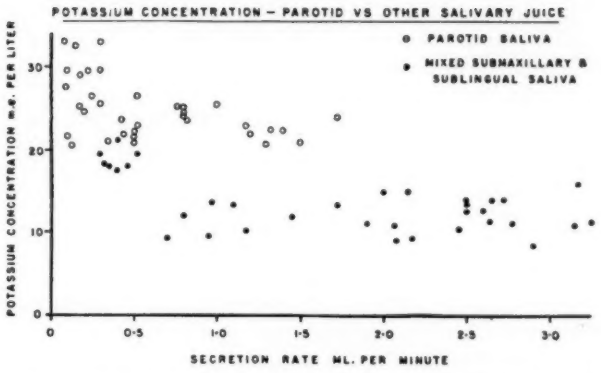

FIG. 3. The potassium concentration in parotid juice compared with that in simultaneously collected mixed saliva from submaxillary and sublingual glands.

All these factors must be taken into account in any study of salivary electrolytes. The dependence of sodium concentration and the independence of potassium concentration on secretory rates makes the sodium–potassium ratio of parotid juice meaningless unless the rate of secretion is known. In addition, the differences in potassium concentration between different salivary glands makes the level of potassium in mixed saliva of little value if relative contributions from the different glands are unknown. There are several reports in the literature (11, 14, 22, 27, 37) on the effect of general disturbances in metabolism such as heart failure, salt depletion, and administration of ACTH, etc., in which the index of the salivary secretion of the electrolytes has been the sodium–potassium ratio of mixed saliva. All this work should be reviewed in the light of the relationship of salivary electrolytes to rates of secretion and to the variations between glands and individuals. This was clearly pointed out by Gregersen and Ingalls in 1931 (15) but appears to have been largely overlooked by subsequent clinical investigators.

The source of salivary electrolytes has been the subject of some discussion in the literature. There is no obvious source other than the blood for the sodium, potassium, and chloride in the amount produced by the stimulated salivary glands. However, the work of Wills and Fenn (39), who stimulated parotid glands both by electrical excitation of secretory nerves and by intravenous pilocarpine injection, is interesting. Both stimuli produced saliva of similar concentrations. However, in the case of pilocarpine stimulation the potassium content of the gland tissue was decreased by approximately 30%, while it remained at the prestimulation level when the gland was caused to secrete by stimulation of nerves. This work suggests that the concentration of potassium in saliva is set at an obligatory level and that, if insufficient potassium is brought to the gland by the relatively reduced blood flow with pilocarpine stimulation, the gland cells go into potassium debt to maintain the obligatory level in the saliva (28).

The salivary bicarbonate could be derived from the blood or it could be a product of the metabolic activity of the gland cells. The latter seems more likely from the work of Sand (31) who found a higher concentration of radioactive bicarbonate in saliva when the radioactive carbon was injected intravenously as lactate than when it was injected as bicarbonate. Also, although labelled bicarbonate given intravenously found its way into the saliva, the specific activity in saliva was lower than that in the blood, again suggesting that at least some salivary bicarbonate was derived from a source other than the blood.

Summary

The differences between the electrolyte secretion by the pancreatic and salivary glands are summarized in Fig. 4. This is a composite figure with data from several sources (1, 5, 7, 16, 28, 32, 36).

Sodium is at a level of approximately 140 meq./liter in pancreatic juice and is fixed at blood level. The source of this sodium is undoubtedly the plasma and changes in plasma are immediately reflected in pancreatic concentration. Salivary sodium varies from 5–100 meq./liter depending on secretion rate and is, as far as we are aware, independent of the blood level. The sodium concentration in gland tissue is not very dissimilar between the two glands, being reported as 45 meq./liter of water in the pancreas and 35 meq./liter in the salivary glands.

Potassium.—Pancreatic juice potassium is again fixed and varies with blood level which is usually between 4–5 meq./liter whereas the salivary potassium is fixed as far as its independence of rate of secretion is concerned but is at a level far higher than blood level. This level varies between the salivary

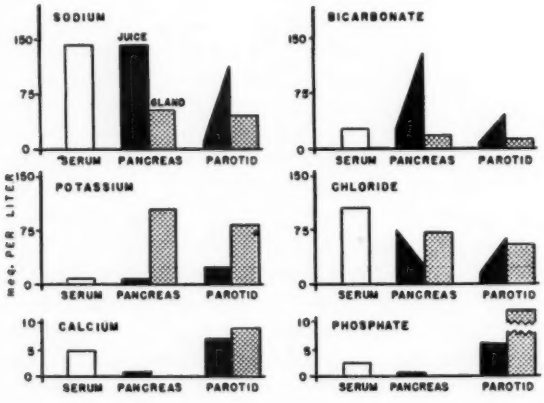

FIG. 4. A comparison between the pancreas and the parotid glands showing the differences in the electrolyte composition of their secretions and the similarities between the electrolyte concentration in the gland tissues. The data for this graph are from several sources.

glands. The cellular concentration of potassium in the pancreas is approximately 105 meq./liter and in the salivary glands approximately 85 meq./liter The high concentration of potassium in saliva seems to take precedence over the glandular concentration.

Chloride concentration in pancreatic juice varies from 75 meq./liter down to 20 meq./liter. The cellular concentration is approximately 70 meq./liter, the juice chloride being inversely related to secretion rate and to the bicarbonate concentration. In the salivary glands chloride concentration increases with rate of secretion but does not achieve blood levels. The cellular content is reported to be approximately 54 meq./liter.

Bicarbonate in the pancreatic juice varies from approximately blood level upwards, depending on secretion rate, reaching an asymptote of approximately 130 meq./liter at rates of secretion above 0.05 ml./min./kgm. Its source appears to be mainly plasma bicarbonate. In contrast, the parotid bicarbonate increases from 5–40 meq./liter with increasing rates of secretion and seems to arise chiefly from the metabolic activity of the gland cells.

The pH of pancreatic juice varies slightly with rate of secretion from 8.0 to 8.3. In parotid juice the pH varies to a greater extent but, like the pancreatic, rises with increasing rate of secretion.

Calcium and phosphate levels in the pancreas are appreciably lower than the blood whereas in the salivary glands they are appreciably higher than the blood.

In short, this last figure summarizes the very considerable differences in the pattern of electrolyte secretion between the isotonic and hypotonic secreting glands exemplified by the pancreatic and the parotid glands. Those who formulate theories concerning the intimate cellular mechanisms of the glandular secretion of electrolytes must account for these differences.

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DISCUSSION: J. G. FOULKS[1]

As a student of electrolyte metabolism, whose interest has largely centered around the homeostatic functions of the kidney, I feel a considerable degree of humility in discussing the function of ions in the secretions of the bowel. In spite of the marked disparity in their ultimate products, these two organs actually share more common properties than is often recognized. Both organs have functions largely concerned with the secretory and reabsorptive transport of dissolved materials. The processes involved are remarkably similar in several instances. For example, the secretion of hydrogen ion is suppressed by carbonic anhydrase inhibition, and the reabsorption of glucose is prevented by phlorizin, in both organs.

In the bowel, the "internal" turnover of fluid (secretion and reabsorption) is on a much more modest scale than in the case of the kidney, whose filtration and reabsorption in the adult human is of the order of 200 liters per day. Nevertheless, the "internal" turnover of fluid in the bowel is substantial (some 6–8 liters per day), and its fluid contents comprise on the average, about 15% of the extracellular fluid volume.

While the kidney bears the major responsibility for homeostasis with respect to water and electrolyte, the bowel is not immune to such influences, although these may involve reabsorption as well as glandular secretions. The well known effects of the adrenal cortical steroids on the transport and distribution of sodium and potassium are not confined to any one tissue. Modifications in the fecal output of sodium and potassium are a well established consequence of altered cortical status. Similar changes are seen in sweat as well as in urine.

However, the glandular secretions of the bowel and its appendages have been adapted to the primary functions of dissolving, digesting, and absorbing various food materials. The rate of the various gastrointestinal secretions is largely adjusted to the intake of food, although salivary secretion is suppressed in dehydration, an adaptation which, by stimulating thirst, promotes restoration of the fluid deficit.

The electrolyte content of the gastrointestinal secretions is not consistently related in any obvious way to over-all homeostatic needs. The relatively fixed electrolyte composition of the pancreatic secretions, with the concentration of most ions reflecting their serum level, permits these secretions to be formed with a minimum amount of net osmotic work or transport against concentration gradients, although this does not preclude active transport. The extension of the concentration of bicarbonate ion at the expense of chloride is the major

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exception. Since bicarbonate is the major buffer of the extracellular fluids, and the carbon dioxide tension tends to be fixed to some extent by pulmonary function in many fluids, flexibility in the concentration of salts of bicarbonate is useful for adjustments in pH. The reciprocal relationship of bicarbonate and chloride, which is also found in the urine, permits the achievement of these anion and pH adjustments without necessitating net osmotic work. However, if the Henderson–Hasselbach equation applies, the high pH (8.0) of pancreatic juice at low secretion rates with a relatively low bicarbonate concentration (35 meq./liter) would seem to preclude complete carbon dioxide equilibration. Perhaps the conversion of carbon dioxide to carbonic acid by carbonic anhydrase in the secretory cells of the gland deprives the secretion of carbon dioxide.

The role which the ionic content of the secretions of the bowel plays in the performance of their functions is still to be clarified in most instances. Most of the hydrolytic ferments have optimal pH requirements, which are presumed to provide substrate ions of the proper charge for their interaction with enzymes, or vice versa. The alternation of these requirements from acid to alkaline in the stomach and small intestine, respectively, permits a substantial pH deviation at each site, while the close temporal sequence of their respective secretions permits this adjustment to be accomplished with minimal disturbance of the acid–base balance of the total extracellular fluid.

The pH requirements of many enzymes are not narrowly defined. Pancreatic amylase, with an optimum pH near 7.8, manifests greater than 50% activity throughout the range from 5.5 to 8.2.

The actual milieu in which the processes of digestion occur frequently consists of a mixture of secretions, and the contribution of a single gland taken in isolation may not give an accurate picture of the functional relationships of the various components.

Certain divalent cations, such as magnesium ion, are known to play an important role in the action of many proteolytic enzymes, possibly by linking substrate and enzyme in chelate rings which bridge and distort peptide bonds and thereby facilitate their hydrolytic cleavage.

In other cases, ions have been implicated in enzymatic processes, while the mechanism of their action remains obscure. Chloride ion for instance is required for amylase activity, and cannot be replaced by other naturally occurring anions such as phosphate or sulphate. This may underlie the fact that in saliva, increase in bicarbonate content with increased secretion rate does not occur at the expense of chloride, as in the case of many other fluids. Potassium ion has been implicated in many enzymatic processes such as the phosphorylation and transport of glucose, the synthesis of protein, as well as in the essential functions of nerve, skeletal, and cardiac muscle. As yet, we can only conjecture as to the type of chemical interactions which are involved in the active transport and enzymatic effects of such ions as potassium, sodium, and chloride.

The salivary secretion has several interesting properties. Its remarkable secretory activity, which can produce a hydrostatic pressure in excess of the blood pressure is well known. The relatively high potassium concentration of saliva, and the fixed obligatory secretion of this ion is remarkable. Gastric juice has also been reported to have a high potassium content. The functional value of this property is not apparent.

Equally obscure is the functional role of the hypotonicity of the salivary secretion. In the case of sweat and urine, hypotonicity permits the conservation of salt from fluids which are lost to the body. Since saliva is completely reabsorbed, the adaptive value of its hypotonicity is more difficult to understand. However, it is unlikely that a process requiring such an expenditure of energy would have evolved without commensurate functional returns on the investment. In general it seems fair to conclude that our understanding of the requirements of various enzyme systems for the major ions of the body fluids, and the mechanisms through which their roles are mediated, is very incomplete and remains a fruitful field for further investigations.

GENERAL DISCUSSION

Dr. B. Grad.—Although I agree in general with the remarks of Dr. Hildes, I take exception to his statement that in many clinical studies the Na/K ratio of the saliva is valueless unless secretion rates are known. Suppose that the sodium concentration was 5 meq. per liter, the potassium concentration 10, and the rate of salivary flow 5 ml. per minute. The sodium output per minute will be 25 μeq. and the potassium 50; the Na/K ratio will be 1/2. With ACTH, the Na/K ratio decreases to, let us say, 4/12. If ACTH increases the secretion rate to 10 ml. per minute, the sodium output will be 40 and the potassium 120 μeq. per minute but the Na/K ratio will still be 4/12, as it was before assuming that the rate had changed. In my opinion, the Na/K ratio in resting saliva provides a valuable indication of the level of adrenal cortical function.

Dr. A. S. V. Burgen.—If one examines the Na/K ratio as a function of rate of secretion in the dog or human parotid secretion, it is found that at low flow rates it may be as little as 0.05, and that it rises with increasing rate of secretion to reach a maximum of 8–10. That is,

the Na/K ratio can normally vary about a hundredfold according to the rate of secretion. If a corticoid is given the curve is displaced downward, so that Dr. Grad is correct in saying that ACTH causes a decrease in the Na/K ratio. However, I think Dr. Hildes' objection to this method of measuring adrenocortical activity is valid, unless the rate and type of secretion are known. At high rates of secretion a maximal effect of corticoids may reduce the Na/K ratio by only 50%, but the same reduction could be produced by a moderate fall in secretion rate without any change in adrenocortical status.

Dr. A. C. Burton.—Because of my particular interest in sweat glands I am sorry that both the speaker and the discussant disregarded them. I had occasion to behave as a catalyst (or inhibitor) in a difference of opinion expressed by Robinson on the one hand, and Weiner at Oxford on the other. Both were studying the relation between the chloride concentration in sweat and the rate of sweat secretion, in hot and cold environments and in exercise. When the rate of sweating rose the chloride ion concentration decreased. Robinson, however, found that the chloride concentration was related to skin temperature. I passed this information on to Weiner who, like a good scientist, spent three days reworking his data. He found that his data, like Robinson's, demonstrated the fact that the chloride concentration of the sweat was determined not by the rate of sweating, but by the temperature of the skin. This investigation of the role of skin temperature resolved their differences which suggests that research on the salivary glands might benefit, too, from some thermodynamic studies.

Dr. B. Grad.—I wish to mention the fact that there is a diurnal variation in the Na/K ratio in resting saliva. The ratio is highest in the morning before breakfast when the secretion rate is lowest. How is one to explain this finding in view of the fact that nervous stimulation of salivary flow results in a higher Na/K ratio ?

Dr. J. A. Hildes.—As the data on Fig. 3 of my paper showed, there is considerable difference in the potassium concentration in saliva from different glands. You do not know the ionic contribution of the different glands to mixed saliva, nor do you know that this contribution is the same at all times.

HORMONAL REGULATION OF BODY ELECTROLYTES

THE ROLE OF THE ADRENAL STEROIDS—A BRIEF REVIEW[1]

By James A. Dauphinee[2]

Introduction

It is now a well established fact in physiology that the concentrations of the individual electrolytes in the body fluids of normal persons are maintained, in spite of wide variations in intake, at essentially constant levels from day to day; and it is equally well established that the maintenance of this constancy is of the utmost importance for the proper functioning of all living cells and tissues.

The preservation of this steady state—in so far as the electrolytes of the extracellular fluids are concerned—is largely the responsibility of the kidneys. Under normal circumstances these organs regulate the excretion of these substances into the urine in such a way that the amounts which are lost from the body by all channels are essentially the same as the amounts which have been taken in. As a result of these balancing activities of the kidneys, both the concentrations and the total quantity of the electrolytes in the body fluids are kept continuously within the relatively narrow, normal physiological range.

The Relation of the Adrenal Cortex to the Over-all Electrolyte Balance

(*a*) *The Effect of Adrenal Insufficiency*

The ability of the kidneys to carry out this very important function is influenced by a great number of different factors but it has long been known to be particularly dependent upon the function of the adrenal cortex.

In conditions of adrenal insufficiency, produced in animals by adrenalectomy or found in human patients with untreated Addison's disease, the concentration of sodium and chloride in the plasma and the total quantity of these elements in the body are both sharply reduced. These abnormalities occur because, as a result of the inadequacy of adrenocortical secretion, the ability of the kidneys to maintain a salt balance on a low or normal intake of salt is impaired and amounts of salt in excess of the intake are excreted into the urine (13). A strongly negative salt balance is thereby established and the body becomes rapidly depleted of its sodium chloride.

Because of the resulting disturbances in osmotic relationships, this loss of sodium ion from the extracellular compartment in adrenal insufficiency is also accompanied by the loss, partly to the urine and partly to the cellular compartment, of an amount of water which is physiologically equivalent to the amount of salt which has been drained away. For this reason the volume of the

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[2] *Recipient with A. G. Cornall of a Grant-in-Aid from the National Research Council.*

extracellular fluid, including of course that of the circulating blood, is correspondingly decreased; and many of the signs and symptoms of this condition—such as the dehydration, hemoconcentration, hypotension, asthenia, elevated bloodnon-protein nitrogen, and others—are explained to a large extent by this loss of sodium and the resulting decrease in the volume of the extracellular fluid and blood plasma, and by the impairment in circulatory efficiency which is associated with these changes. That this is so is shown by the fact that many (but not all however) of the clinical manifestations of this condition can be considerably relieved by the administration of large amounts of sodium chloride without any other treatment (12).

In addition to the excessive renal loss of sodium and the consequent fall in the plasma sodium concentration there is also often in this condition a decrease in the excretion of potassium and an increase in the concentration of this ion in the blood and extracellular fluids (34).

Similar though less marked and important changes also take place in the content of both sodium and potassium in the sweat (2), saliva (7), and gastrointestinal secretions (30) in individuals with adrenal insufficiency and the extent of these changes is sometimes of value in helping to appraise the level of adrenocortical activity.

(*b*) *The Effect of the Administration of Adrenocortical Extracts and Adrenocortical Hormones*

(*1*) *To Individuals With Adrenal Insufficiency*

The excessive loss of urinary sodium, the renal retention of potassium, and the other electrolyte and metabolic abnormalities which occur in adrenal insufficiency are all corrected by the administration of adequate amounts of potent extracts prepared from normal adrenal glands. From such extracts some 29 or more substances of steroid nature have been isolated in crystalline form. Many of these are physiologically inert but a few of them have been shown to possess biological activities which indicate that one or more of them are probably responsible, at least in part, for the potency of the crude preparation. The chemical structure of some of these active materials is given in Fig. 1.

Of the compounds shown in Fig. 1 the most effective, per equivalent unit of weight, in correcting the renal loss of sodium and the retention of potassium in patients with Addison's disease, is desoxycorticosterone; and in human patients the regular administration of doses of 1–3 mgm., or occasionally up to 5 mgm., of this compound daily (30), along with moderate amounts of added salt, will usually be sufficient to restore the defective electrolyte metabolism to normal and to correct the clinical disturbances which are due to salt loss. Overtreatment of these patients, however, has to be carefully avoided because in many cases the continued administration of more than the necessary amounts of desoxycorticosterone and salt may lead to the retention and accumulation of abnormal quantities of sodium chloride and water in the body and to definite clinical edema, hypertension, and even to cardiac insufficiency (6).

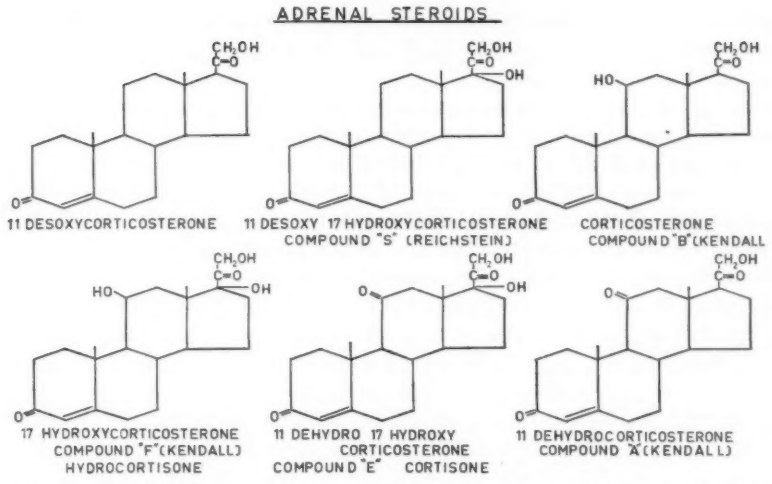

FIG. 1. Chemical structure of some physiologically active steroid compounds which have been isolated from the adrenal cortex.

The other adrenocortical hormones, as well as a number of other steroid compounds, also possess some sodium retaining activity although most of them are very much less powerful in this respect than desoxycorticosterone (see Thorn *et al.* (30) for references). The potencies of these compounds in this regard are commonly compared with that of pure synthetic desoxycorticosterone acetate and the results of one such series of comparisons, reported by Simpson and Tait (24), are given in Table I.

TABLE I

COMPARATIVE MINERALOCORTICOID ACTIVITY OF STEROID HORMONES*

Hormone	Other designation	Relative mineralocorticoid activity per unit mass
Desoxycorticosterone	DOC	135
Desoxycorticosterone acetate	DOCA	100
Corticosterone	B	14
11-Desoxy-17-hydroxycorticosterone acetate	S	8
17-Hydroxycorticosterone (hydrocortisone)	F	7.5
11-Dehydrocorticosterone acetate	A	6.7
11-Dehydro-17-hydroxycorticosterone	E	5.9
11-Dehydro-17-hydroxycorticosterone acetate	EAc	5.2
Estradiol		3
Progesterone		3
Testosterone		1.5

* *Table prepared from data of Simpson and Tait (24).*

(2) *To Normal Individuals and to Patients Without Adrenal Insufficiency*

Not only can desoxycorticosterone and the other related adrenal steroids decrease the sodium chloride excretion and increase the elimination of potassium in patients and animals with adrenal insufficiency but they tend also to influence the excretion of these electrolytes in the same direction when they are administered to normal individuals (see Thorn *et al.* (30) for references). The size of the dose, however, which is necessary under these circumstances to produce a definite retention and accumulation of sodium, as well as an increased excretion of potassium, is usually considerably higher than it is in comparable individuals with adrenal insufficiency.

In addition, the effect of these compounds upon the electrolyte excretion of normal individuals is often of only a temporary nature, and when they are administered for a period longer than a week or 10 days there is frequently, at least as far as sodium and chloride are concerned, a pronounced "escape" from the initial response. In such cases the characteristic early retention is frequently followed by a period of increased urinary output so that a normal or even negative balance of salt is soon attained (20). Patients suffering from the edema of lipoid nephrosis often show, for example, after a short period of retention, a pronounced water and salt diuresis and a decrease in the severity of their edema when they are treated with cortisone, hydrocortisone, or ACTH (15); and dogs, subjected to long continued treatment with large (25 mgm. /day), or even moderate (2–4 mgm./day) (18), doses of desoxycortisone develop a diabetes insipidus like syndrome in which the severity of the diuresis is proportional to the amounts of sodium chloride which are being given to the animals at the same time.

In rats on the other hand the prolonged administration of large doses of desoxycorticosterone and excessive amounts of salt has been shown to produce severe hypertension and hypertensive vascular and renal disorders (23). In man persistent treatment with large doses of cortisone may occasionally lead to hypertension, particularly in the presence of chronic renal disease (19) and it may cause the appearance of a hypochloremic, hypokalemic alkalosis (30) similar in its nature to that which is occasionally encountered in patients who are exhibiting the effects of hyperadrenalism in Cushing's syndrome (32).

(*c*) *Aldosterone*

Although small amounts of desoxycorticosterone have been found in some adrenal extracts many investigators have felt that this steroid is not a normal or natural product of the adrenal gland. On the other hand all potent adrenocortical preparations, including the so-called "amorphous fraction" remaining after the crystallizable hormones have been removed, always possess a powerful sodium retaining activity, and this has been found to be much greater than could be accounted for, until recently, by the other steroids known to be present. For this reason considerable effort has been made by many investigators to isolate and identify the natural substance present in

the adrenal cortex which is responsible for the powerful sodium retaining activity exhibited by these extracts and by other preparations made from normal adrenal glands.

A partial purification of this principle was accomplished by Thatcher and Hartman in 1946 (28) and the substance so obtained was shown not only to be much more powerful than desoxycorticosterone in regard to its ability to cause sodium retention but also to differ from the synthetic hormone in many of its other properties. Finally, in 1952, Grundy, Simpson, and Tait (8) isolated from potent adrenocortical extracts by paper chromatography a steroid fraction which has been shown to have from 25 to 100 times the mineralocorticoid activity of desoxycorticosterone. The same substance has been obtained by others in an amorphous state or in crystalline form and its structural formula, which is given in Fig. 2, has recently been established by Simpson and Tait working in Reichstein's laboratory (25). Known as "electrocortin" before its chemical structure was determined this specific steroid hormone has now been given, because of the presence of the aldehyde group at C_{13}, the partially descriptive name of "aldosterone" (25).

Although, as has been indicated, a wide variety of potent steroids have been isolated from extracts of the adrenal cortex it seems likely, from the investigations of the hormone content of blood perfused through the adrenal gland *in situ* and also of peripheral blood, that the principle steroids which are normally elaborated by the adrenal cortex are 17-hydroxycorticosterone and corticosterone along with the above mentioned aldosterone (1, 26). Of these, 17-hydroxycorticosterone or compound F is most active in regard to its effect on carbohydrate and protein metabolism, but it also possesses, particularly when given intravenously, a very definite sodium retaining activity (29). On the other hand, aldosterone, which possesses from 25–100 times the sodium retaining activity of desoxycorticosterone, has also been shown to affect carbohydrate metabolism as well. By the glycogen deposition test, for example, it has been found to be at least 30 times as potent as desoxycorticosterone in causing glycogen to be deposited in the liver of the adrenalectomized rat, although in this respect it is only one third as active as cortisone (22).

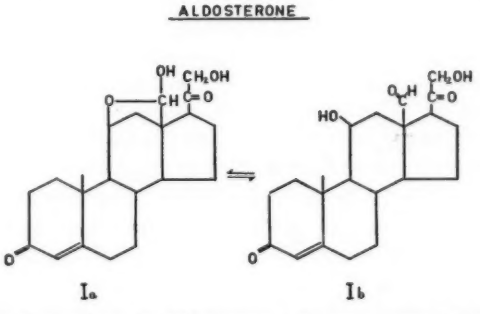

FIG. 2. Chemical structure of Aldosterone, the very powerful mineralocorticoid recently isolated from the adrenal cortex (25).

In the adrenal vein blood about 60% of the sodium retaining effect has been found to be due to aldosterone and the remainder is probably due to the combined effects of 17-hydroxycorticosterone and corticosterone (26).

(d) Mineralocorticoid Activity in the Urine

Of great interest in this connection are the observations that have recently been made concerning the presence, in the urine of animals and humans under varying conditions, of an extremely potent sodium retaining factor which has been shown to possess, as far as can be determined, the physical, chemical, and pharmacologic properties of aldosterone, and which is probably identical with this hormone (16).

It has been found, for example, that when the need to conserve salt is increased, as it is when an individual is placed on a low salt intake, the content of sodium retaining factor in the urine is increased. It has been found too that when edema or ascites is accumulating, and salt is being retained, in such pathological states as myocardial insufficiency, lipoid nephrosis, and cirrhosis of the liver, the sodium retaining activity of the urine may be elevated to levels which are many times above the normal values (see Luetscher and Johnson (16) for references). When however in these cases the rate of salt excretion becomes much increased and the edema is disappearing the concentration of this mineralocorticoid in the urine falls back to normal or to less than normal values.

It is probable that these urine findings reflect, to some degree at least, the changes in the amount of this hormone which is in the circulating blood and, if this is so, it suggests that when there is a need to conserve salt by decreasing its excretion, some kind of stimulation leads to the formation or release of increased amounts of this potent sodium retaining material. It also suggests that in certain pathological conditions in which edema is being accumulated this hormone may play a part and be the cause of an active retention of salt by the kidney. Whether this is a primary factor in the causation of edema or whether it is secondary to the need to conserve salt because this mineral has been drained away from the circulating plasma into the interstitial spaces is a question which still needs further clarification.

It would appear that this potent salt retaining urinary hormone is elaborated by the adrenal cortex, for it is absent from the urine of patients whose adrenals have been damaged by tuberculosis, or other causes of Addison's disease, or who have undergone bilateral adrenalectomy (14). On the other hand it is present in detectable amounts in the urine of normal children and adults, and also in that of individuals who have well marked pituitary deficiency (14). These latter results suggest that a part, at least, of the sodium regulating function of the adrenal cortex, in contrast to its other functions, is largely independent of the influence of the anterior pituitary and they agree of course with the observation that hypophysectomized animals, with intact adrenals, show no evidence of excessive salt loss or need for extra salt (5) and also with other indirect anatomical and clinical evidence which

points in the same direction. They also agree with the recently reported finding that whereas the 17-hydroxycorticosterone content of the plasma is very definitely increased by the administration of ACTH, the aldosterone concentration is not significantly affected by this treatment (26), and with the observation that whereas the 17-hydroxycorticosterone content of the blood is reduced to zero by hypophysectomy this procedure decreases the aldosterone concentration by only a moderate degree (27). On the other hand Venning and co-workers (31) have reported that the aldosterone content of the urine is increased by the administration of growth hormone, a finding which suggests that the production of this salt retaining compound may not be entirely free from central pituitary control.

(*e*) *Salt Retention in Stress*

A definite suppression of salt secretion, leading to an active retention of this mineral, is well recognized to be among the characteristic responses to the stressful effects of surgical operations or of other trauma (17). As this effect is almost certainly mediated through the action of the anterior pituitary it is probable that the salt retention which occurs in these cases, as well as that which occurs in patients treated with ACTH, is due, not to the action of aldosterone, but rather to the marked increase in the amount of salt-retaining 17-hydroxycorticosterone which has been shown to be produced by the adrenal under these circumstances (29).

The Relation of the Adrenocortical Hormones to Renal Function

So far in this discussion attention has been paid mainly to the effects of the adrenocortical hormones on the excretion of salt by the kidney and on the over-all balance of salt in the body, and little consideration has been given to the renal mechanisms whereby these effects are achieved. In adrenal insufficiency, as has been stated, the sodium and chloride excretion by the kidney is increased and the potassium excretion is decreased. As it has been definitely shown that the rate of glomerular filtration is considerably diminished in this condition (10) it follows that the excessive sodium and chloride excretion of the adrenal deficient state must be due to some kind of tubular disturbance which results in an imperfect or inadequate reabsorption of these ions from the glomerular filtrate by the cells of the renal tubular epithelium.

The correction of the excessive renal excretion by the administration of desoxycorticosterone or other adrenal steroids is on the other hand associated with an increased glomerular filtration rate (30) and it is therefore obvious that these hormones must decrease the renal excretion of sodium by increasing the rate or extent of tubular reabsorption.

Although it is generally agreed that in adrenal insufficiency the imperfect tubular reabsorption of sodium and chlorine is due to an inadequate production of adrenocortical steroids, there is as yet no precise knowledge about the true nature of the tubular defect which is caused by such a lack nor about the mode

of action of the missing hormones. It is possible that adequate amounts of these substances must be present to permit the tubular cells to bring about the almost complete reabsorption of these ions from the tubular fluid which is required for salt conservation when the salt intake is low; or that they are needed for the tubular formation and secretion of ammonium or hydrogen ions which, when necessary, are normally called upon for the conservation of fixed base (21). Another possibility is that in the absence of these essential adrenocortical factors there is established a condition of hormonal imbalance which permits the unopposed natriuretic and chloruretic activities of the posterior pituitary secretion to predominate. Some further remarks regarding the role of the posterior pituitary in regulating electrolyte metabolism will be made by Dr. Noble in the discussion which follows this paper.

The inadequate tubular reabsorption of sodium and chloride in adrenal insufficiency, leading to an excessive excretion of these ions into the urine, can probably be considered to be a primary result of the deficient production of the salt retaining hormones in this condition. It is probable, too, that the depressed ability of the tubules to form and secrete ammonium and hydrogen ions is also in the same category because, in salt-maintained, adrenal deficient animals who have a normal filtration rate and renal plasma flow, the defective ammonia production can be restored to normal by the administration of desoxycorticosterone or potent adrenocortical extracts (21).

In addition to these tubular abnormalities, however, the adrenal deficient individual also exhibits a sharply diminished renal plasma flow and glomerular filtration rate (10), an elevated blood non-protein nitrogen and potassium concentration, and other evidences of impaired renal function. Although the defective excretion of potassium may also be, in part at least, a primary result of inadequate hormonal control of tubular activity, it is probable that many of the other renal disturbances are not primary manifestations of a specific hormonal lack but are rather results which are secondary to the hemodynamic upsets brought about by the decrease in the volume of the circulating blood, which is produced, as has been mentioned, by the excessive loss of sodium ions.

The Relation of the Adrenocortical Hormones to the Distribution of Electrolytes Between the Cellular and Extracellular Compartments

Not only do the steroid hormones of the adrenal cortex help to regulate the renal excretion of the electrolytes and the over-all balance of these substances in the body but they also influence to a considerable degree the relative distribution of these electrolytes between the cellular and extracellular compartments.

In untreated adrenalectomized animals, for example, the muscle sodium falls and the muscle potassium becomes considerably elevated (9), and these abnormalities can be corrected or prevented by the administration of adequate amounts of potent adrenocortical extracts, desoxycorticosterone, or other adrenocortical steroids. When given to normal individuals the administration of desoxycorticosterone causes, both in experimental animals and in man, a

definite increase in muscle sodium and a marked decrease in muscle potassium (3). Similar results have been shown to occur after the administration of sufficiently large doses of cortisone (see Thorn *et al.* (30) for references) and in both cases the magnitude of the electrolyte change is probably also influenced to a considerable degree by the absolute and relative intake of sodium and potassium salts.

Whether these effects are merely secondary to the results of the action of these hormones on the renal excretion of electrolytes or whether they occur because of a direct action on the cells of muscle and other tissues is a question which is not entirely settled. The evidence indicates that these hormones can influence directly the electrolyte content of sweat (2) and saliva (7) and it has been demonstrated that the electrolyte absorption of the gastrointestinal tract is considerably slowed by adrenalectomy and is restored by the administration of adrenocortical hormone (4). These observations along with those of Levitt and Bader (11) on the effect of cortisone and ACTH on the electrolyte distribution in humans maintained on a salt free diet, and of Woodbury (33) on the effect of adrenocortical hormones on the distribution of electrolytes in nephrectomized rats, indicate that it is likely that "the adrenocortical hormones influence the electrolyte metabolism in all the tissues of the body and that this influence is exerted independently of the known effects of these hormones on the kidney" (33).

Summary

The steroid hormones of the adrenal cortex play a very important part in the regulation of the sodium and chloride excretion by the kidney and of the over-all balance of electrolytes in the body. They probably, too, are of considerable importance in regulating the distribution of the electrolytes between the cellular and extracellular tissues. No conclusive evidence is as yet available to indicate the nature of the fundamental mechanisms whereby these effects are brought about.

These hormones can be divided into two separate categories. In the first category are corticosterone and 17-hydroxycorticosterone (hydrocortisone, hydrocortone, cortisol, or Compound F) the secretion of which is governed by the activity of the anterior pituitary gland and whose mineralocorticoid activity is relatively slight. In the second is the newly identified aldosterone, a very powerful salt retaining hormone, whose production and release would seem to be largely independent of anterior pituitary control and which, through some as yet undetermined mechanism, is set free in increased amounts whenever there is present a need—or a situation which is interpreted as a need—for the retention of sodium.

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DISCUSSION: R. L. NOBLE[1]

It has been suggested that I might add to Dr. Dauphinee's comprehensive summary of the adrenal influence on electrolytes a brief discussion on the possible role of the posterior pituitary gland in maintaining electrolyte balance in the body. Any such discussion logically centers around, not only the effects of posterior lobe hormones, but also the more speculative interrelationship between the adrenal cortex and the neurohypophysis. (This is a big field, Mr. Chairman, and I think it requires six rather than the alloted five minutes.)

The typical action of the posterior lobe hormones on urine formation is well known. The antidiuretic hormone which is synonymous with the vasopressive hormone causes a marked increase in the reabsorption of water by the kidney tubule, resulting in the excretion of a concentrated urine. This action is typically demonstrated after the administration of an ingested water load which would normally provoke a prompt diuresis. Studies by Van Dyke

[1] *Contribution from Collip Medical Research Laboratory, University of Western Ontario, London, Canada.*

and associates using the pure and also synthetically prepared hormones of Du Vigneaud and collaborators have shown that the oxytocic hormone also possesses antidiuretic activity to perhaps five per cent of that of the pure antidiuretic substance. Total deficiency of posterior lobe hormones is associated with the inability of the kidney tubules to reabsorb water with a resulting copious excretion of unconcentrated urine. The induction of such a condition of diabetes insipidus is dependent on the destruction of certain nuclei of the hypothalamus to cause degeneration and cessation of all hormone production, since removal of the posterior pituitary alone is inadequate. In addition, the typical symptoms of the condition are dependent on a normal function of the anterior pituitary. Removal of this gland causes an immediate cessation of the polyuria of diabetes insipidus. The effects of simple hypophysectomy on water metabolism are therefore complex, and apparently are not related to secondary adrenal atrophy. Usually only a transient polyuria follows the operation. The ability to excrete a water load in normal fashion, however, is rapidly lost. This characteristic of the hypophysectomized animal is similar to the findings in adrenal insufficiency although it is probable that the mechanisms differ.

These described effects of posterior lobe hormones have concerned primarily the water balance of the body and only affect electrolytes indirectly. Unfortunately, for the purposes of this discussion any direct action on electrolytes is of doubtful physiological significance. It is true, however, that posterior lobe hormones may under some conditions, such as when given to animals under anesthesia, cause an increased urine output and an increased excretion of sodium and chloride in the urine. This effect is exaggerated by an increased salt intake. The McGill workers in the Department of Pharmacology have favored the view that salt mobilization by posterior lobe hormones, possibly oxytocin, causes the diuretic action.

Extensive studies in recent years have shown that posterior lobe secretion may be stimulated under a variety of conditions. Admittedly, some of this evidence stems from the finding of a provoked excretion of an unidentified antidiuretic substance in the urine, yet the circumstantial evidence is strong that such a principle is the antidiuretic hormone. Factors such as dehydration, or the injection of hypertonic saline causing an increased osmotic pressure in the blood, according to the concept of Verney and associates results in the stimulation of osmoreceptors and hypothalamic activation of the posterior lobe. This mechanism probably represents the chief form of physiological control. In addition, stimulation may also follow emotional reactions, fainting, electroconvulsive therapy, nicotine absorbed from cigarette smoking, etc. Little acceptable evidence is available of diseased states causing a direct overproduction of posterior lobe hormones. From the osmotic regulatory mechanism it might be anticipated that the adrenal cortex in its ability to influence electrolyte levels should indirectly affect posterior lobe secretion. Also there is evidence of a direct effect of adrenal hormones on the neurohypophysis; from the unconfirmed isolated observation that cortisone treatment of the rat may result in demonstrable degenerative lesions in hypothalamic nuclei. Less direct evidence exists that the neurohypophysis may exert a controlling influence on adrenal function. Conditions, however, which stimulate posterior lobe secretions in most cases result simultaneously in a discharge of ACTH by the anterior pituitary. Some observers have actually considered that neural hormones may act as a direct local stimulus to ACTH liberation. Finally, there are the interesting observations that epinephrine, which may also be liberated under the previously mentioned conditions, tends under some circumstances to inhibit the secretion of posterior lobe hormone, but conversely to stimulate ACTH production and hence the adrenal cortex.

Into this background of speculative possibilities of adrenal, neurohypophyseal interrelationships has recently been precipitated a mass of new data obtained from measurements of an antidiuretic substance in blood. Perhaps prematurely this activity has been regarded as due to posterior lobe hormone, although such a view is not yet established. The surprisingly large amounts of antidiuretic activity in rat serum originally described in 1949 by Birnie and associates have now been suggested as resulting from neurohypophyseal stimulation due to the associated repeated bleedings (Ginsburg 1954). With standardized procedures, however, it does seem that the blood of adrenalectomized animals exhibits greater antidiuretic potency than does that of intact rats. Treatment of the adrenalectomized animal with salt or cortisone prevents such a discrepancy. Ginsburg has also shown that injected antidiuretic hormone disappears more slowly from the blood of the adrenalectomized rat. Treatment with cortisone but not salt lessened this slowed rate of disappearance. The hypophysectomized animal has been repeatedly shown to exhibit a reduced amount of antidiuretic substance in the blood. However, Minsky and collaborators have recently found that if subsequent adrenalectomy is performed there is a sharp rise in plasma antidiuretic activity. This may indicate an extra hypophyseal source of this substance, although it is possible that it may be secreted from the hypothalamus. However, the need for comparable studies on rats with diabetes insipidus is obvious.

These results, if considered from the adrenal neurohypophyseal relationship, would suggest therefore that the antidiuretic substance is more readily liberated after hemorrhage in the adrenalectomized animal, remains longer in the blood, and is less readily excreted by the

kidney. Such findings are in harmony with the general effects of adrenalectomy on slowing down metabolic processes, but they should also be considered in terms of the removal of the hormone of the adrenal medulla.

Obviously, it will not be possible for me to conclude with one of the expected charts showing a dissected adrenal opposite a dissected pituitary with the blue and red arrows darting in and out of a kidney tubule, and ultimately pointing to sodium and chloride. There is perhaps some evidence of opposing actions of these endocrine glands on electrolytes, but the over-all picture remains predominantly one of the different adrenal cortical zones controlling electrolytes via a kidney mechanism, and the neurohypophyseal secretions affecting the same organ but primarily influencing water balance.

This review is based on the following papers:

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GENERAL DISCUSSION

Dr. J. C. Laidlaw.—Dr. Dauphinee has implied that the condition of adrenocortical insufficiency leads to an unremitting loss of salt by the kidney. That the situation is somewhat more complex than this was illustrated in a study carried out in Dr. G. W. Thorn's laboratory (Arons, Nusimovich, and Thorn, unpublished) on a male patient in a most severe Addisonian crisis. To our surprise this man was not in negative sodium balance on an intake of approximately 30 meq. per day. Moreover, the value for the total exchangeable body sodium was within the normal range despite the finding of a serum sodium level of 121 meq. per liter. The blood urea nitrogen level was 180 mgm. % indicating severe renal impairment with probably a greatly diminished glomerular filtration rate. While there is little doubt that, in the early stages of adrenal insufficiency in this patient, renal salt loss had occurred, in the later stages of the condition it is probable that progressive salt loss had been slowed down to a considerable degree by the marked fall in glomerular filtration rate. Because of the state of the kidney in this particular case the salt loss was much less than might be expected in Addisonian crisis.

Dr. K. A. C. Elliott.—Since there is some uncertainty about a direct effect of the adrenal steroids on tissues I might mention that Dr. Pappius and I have observed that when DCA is put in the medium there is a decrease in the potassium of brain slices.

Dr. R. A. Cleghorn.—Restoration of the electrolyte balance in adrenal insufficiency by desoxycorticosterone will not, in itself, lead to correction of the abnormal electroencephalogram of patients with Addison's disease. A C-11 oxygenated steroid is necessary to effect that change.

Dr. E. H. Venning.—Dr. Dauphinee has suggested that I discuss some of our findings regarding the influence of the anterior pituitary on aldosterone secretion. When ACTH was administered to normal individuals no increase in excretion of aldosterone was observed. In four normal males the administration of growth hormone caused an increased output of aldosterone. These experiments were carried out a few years ago and the preparations of growth hormone available at that time may not have been pure. We intend to repeat the experiments as soon as more potent preparations are available.

Dr. J. G. Foulks.—In estimating the mechanism of action of hormones on electrolyte balance and distribution, it may be useful to stress the interrelationships between the various hormones and the various ions involved. There is considerable evidence to the effect that potassium retention in adrenal insufficiency is secondary to the defect in the disposition of sodium, since it can be prevented by maintenance of an adequate sodium intake. The present experimental evidence suggests that the primary mechanism for the excretion of potassium is its secretory transport by the renal tubule. This process appears to be activated by cellular hypertonicity. Unpublished experiments performed in the laboratory of Dr. Alfred Gilman have shown that adequate sodium infusion in the adrenalectomized dog will produce a normal renal secretory response. The failure of the kidney to excrete potassium in normal amounts in the Addisonian may therefore reflect the hypotonicity of the body fluids secondary to loss of sodium. In hypercortical states, the prevention of potassium depletion by restriction of sodium intake provides additional evidence that the adrenal corticoids do not directly alter the renal tubular transport of potassium. The prevention of sodium retention by increased potassium administration in cortisone-treated experimental animals further illustrates the

importance of these ionic relationships and the dependence of the hormonal effects on the pattern of electrolyte intake. Similar relationships have been shown between acid–base balance and potassium disposition in altered adrenal cortical states.

While the actions of the antidiuretic hormone are primarily manifested in the over-all control of water balance, the nature of the effect on renal tubular water transport has not been firmly established. In the absence of antidiuretic hormone, water diuresis involves the active reabsorption of solute free of its normal osmotic complement of water. The poor water diuresis which characterizes adrenal insufficiency may be related to the defect in tubular electrolyte reabsorption rather than to a direct effect on water transport mechanisms.

Dr. B. Grad.—If aldosterone is not under control of the anterior pituitary and if the sugar-active hormones are, how can we explain sodium retention and potassium excretion following ACTH ?

Dr. J. A. Dauphinee.—I should like to ask Dr. Laidlaw to answer this question. I suggest that the retention of sodium following ACTH was not due to aldosterone but to the liberation of hydrocortisone (Compound F) which is probably sufficient in amount to account for the salt retention observed.

Dr. J. C. Laidlaw.—I agree with Dr. Dauphinee. When given intravenously Compound F is just as effective in its control of electrolyte metabolism as DCA; it is much less effective, however, when given intramuscularly or by mouth. Since ACTH stimulates the production of Compound F by the adrenals, the injection of ACTH simulates the intravenous administration of Compound F.

Dr. R. A. Cleghorn.—In studying the effects of these hormones or of any drugs the route of administration is very important.

Dr. K. Savard.—We have considerable information on the effect of ACTH on the adrenals of many species but only a limited knowledge of its action on human adrenals. Human adrenal venous blood is known to contain physiologically significant amounts of corticosterone (Compound B) which has considerable salt-retaining activity. Hence when considering the over-all picture, the findings cannot be entirely interpreted in terms of hydrocortisone (Compound F) alone.

Dr. J. A. Dauphinee.—Species differences are very great with regard to the relative amounts of hydrocortisone and corticosterone in adrenal venous blood. This has been shown in a recent paper by Bush (J. Endocrinol. 9 : 95. 1953). For example, in the rabbit corticosterone predominates whereas in the monkey the reverse is true.

Contents—Concluded

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